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# PHYSICOCHEMICAL STUDIES ON THE CATALYTIC AND REGULATORY SUBUNITS OF RIBONUCLEOTIDE REDUCTASE FROM CALF THYMUS

ROBERT JOSEPH MATTALIANO

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SUBUNITS OF RIBONUCLEOTIDE REDUCTASE FROM CALF THYMUS

*University of New Hampshire*

Ph.D. 1982

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SUBUNITS OF RIBONUCLEOTIDE REDUCTASE FROM CALF THYMUS

BY

ROBERT JOSEPH MATTALIANO  
B.S., Stonehill College, 1977

A DISSERTATION

Submitted to the University of New Hampshire  
in Partial Fulfillment of  
the Requirements for the Degree of

Doctor of Philosophy  
in  
Biochemistry

December 1982



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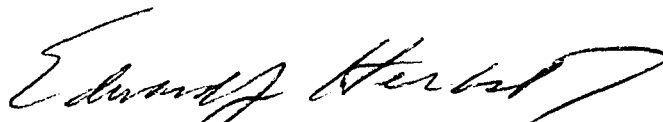
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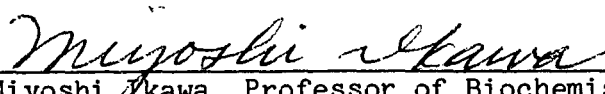
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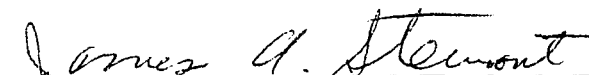
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To my Mother and Father,  
for continuing support and encouragement.

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## ABSTRACT

### PHYSICOCHEMICAL STUDIES ON THE CATALYTIC AND REGULATORY SUBUNITS OF RIBONUCLEOTIDE REDUCTASE FROM CALF THYMUS

by

ROBERT JOSEPH MATTALIANO

University of New Hampshire, December 1982

The catalytic (M2) and regulatory (M1) subunits of calf thymus ribonucleotide reductase have been purified to homogeneity. Each runs as a single band in SDS-polyacrylamide gel electrophoresis with polypeptide molecular weights of 84,000 for M1 and 58,000 for M2. Additional evidence for homogeneity was obtained using high performance liquid gel chromatography. The native M1 subunit is predominately monomeric ( $R_s = 37$  A) under experimental conditions while the M2 subunit is oligomeric ( $R_s = 53$  A). The quaternary structure of the native M2 protein was evaluated by chemical cross-linking with dimethyl suberimidate. The results demonstrate that native M2 protein exists in a dimer-tetramer equilibrium. The amino acid composition of the complementary subunits of the ribonucleotide reductase has been determined. Significant compositional homology exists between the M1 polypeptide and its E. coli (B1) counterpart. However, comparative analytical peptide mapping reveals that the M1 and B1 polypeptides have different primary structures.

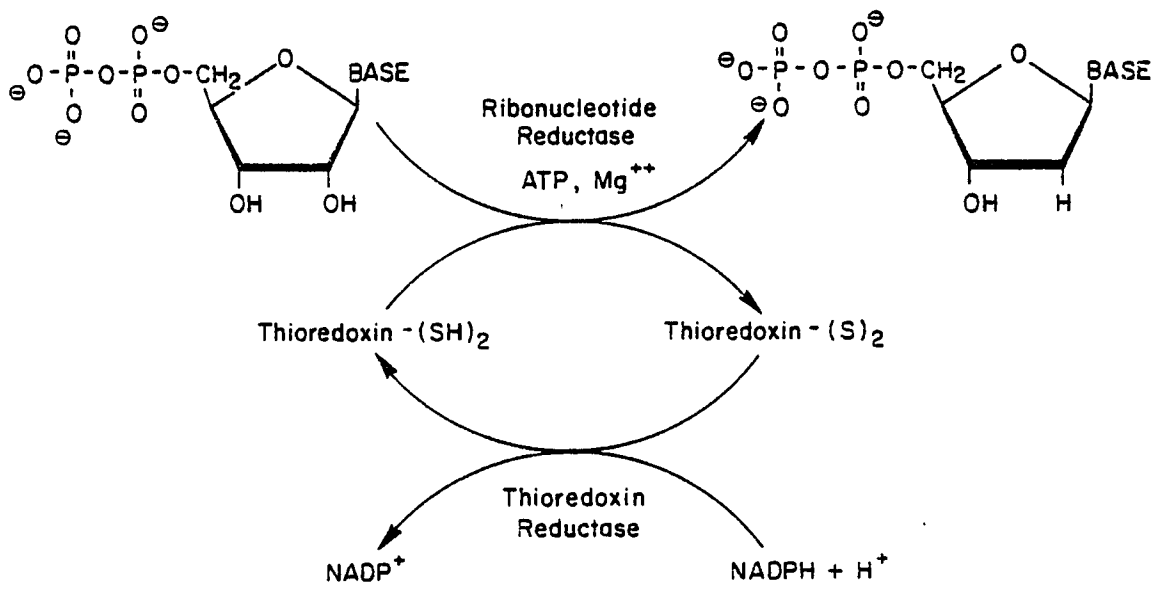
Atomic absorption analysis indicates that protein M2 contains 3 g-atoms Fe/mole monomer, in contrast to the 1 g-atom Fe/mole monomer reported for protein B1. The role of protein M2 associated iron is not yet clear, since addition of exogenous iron to ribonucleotide reductase preparations results in a nearly two-fold enzyme activation. The M2 subunit has a UV-visible absorption spectrum with a major peak at 404 nm but this spectrum is different from that of the B2 subunit of E. coli ribonucleotide reductase. This chromophore is also observed in intact calf thymus enzyme preparations. Addition of ATP, a positive allosteric effector, to intact enzyme results in an increase in the extinction coefficient of the visible chromophore. This spectral enhancement may reflect a shift in equilibrium toward an active protein M1-M2 complex and/or a structural change at the chromophore. EPR spectroscopic experiments at 77 K did not detect a free radical species in the calf thymus enzyme analogous to that which occurs in the E. coli ribonucleotide reductase. Thus, significant differences between the ribonucleotide reductases from these eukaryotic and prokaryotic sources are indicated.

## I. INTRODUCTION

The conversion of ribonucleoside diphosphates to the corresponding 2'-deoxyribonucleotide is catalyzed by ribonucleotide reductase in combination with thioredoxin, thioredoxin reductase, and NADPH. The ribonucleotide reductase system also requires the presence of a positive allosteric effector (ATP) and other cofactors (adenosylcobalamin or  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ). Thioredoxin, a protein which contains a dithiol, serves as a source of reducing equivalents during the catalytic process. It is recycled by the action of the flavoprotein, thioredoxin reductase. The ultimate hydrogen donor for the ribonucleotide reductase system is NADPH (Figure 1).

The reduction of ribonucleotide precursors is a critical and rate-controlling step in the DNA synthetic process and ultimately cell division. Ribonucleotide reductase activity has been found to parallel the rates of DNA replication (Turner et al., 1968) and cell proliferation in the development of invertebrates (Noronha et al., 1972; de Petrocellis and Rossi, 1976), amphibians (Tondeur-Six et al., 1975), and mammals (Elford et al., 1970; Hopper, 1972; Millard, 1972; Cory, 1975). For cultured mammalian cells, the activity of ribonucleotide reductase has in addition been found to be related to that fraction of the cell population synthesizing DNA, e.g., cell cycle dependent (Turner et al., 1968; Murphree et al., 1969).

Figure 1. The ribonucleotide reductase system.





The first indication of a mechanism for the production of deoxyribonucleotides directly from ribonucleotides was the result of an investigation performed using rats injected with [ $^{15}\text{N}$ ]-cytidine (Hammerstein et al., 1950). Significant incorporation of label occurred not only in RNA but also in DNA pyrimidines. This result suggested the existence of a conversion mechanism of ribose to deoxyribose when the sugar contained an N-glycosidic linkage and was consistent with the observation (Bendich, Getler, and Brown, 1949) that free cytosine could not be utilized directly in DNA synthesis. Confirmation of this hypothesis was obtained using ribonucleoside precursors which contained separate radioisotope labels in the base and sugar moieties (Rose and Schweigert, 1953). Nucleosides labeled with radiocarbon were isolated from Euglena gracilis grown on  $^{14}\text{CO}_2$ . After purifying the labeled cytidine from algal extracts, the base and sugar moieties of the nucleoside were separated and their respective specific activity determined. It was demonstrated that totally labeled cytidine, after incorporation into rat DNA or RNA, had the same specific activity ratio as that prior to injection into the animal. This result indicated that 1) the incorporation of pyrimidine nucleosides into RNA and DNA occurred without prior cleavage of the glycosidic linkage and 2) a mechanism existed for the conversion of nucleosidyl ribose to deoxyribose. The later discovery of an enzyme, ribonucleotide reductase, in extracts of E. coli (Reichard et al., 1961) capable of converting cytidine diphosphate to the corresponding deoxyribonucleotide clarified the second observation mentioned above.

During the two decades from the early 1960's to the present, the

enzymatic reduction mechanism for ribonucleotides and the proteins which catalyze this reaction have been intensely studied (Reichard, 1968; Thelander and Reichard, 1979). Ribonucleotide reductase systems appear to be ubiquitous in nature and have been observed in numerous tissue types from different taxonomic kingdoms.

Ribonucleotide reductase has been most extensively studied in two microorganisms, Eschericia coli and Lactobacillus leichmannii. The enzymes from these sources exemplify the two major classes of ribonucleotide reductases. Other types as well have been observed and are also listed in Table I. Although these enzymes catalyze the same reaction, individual enzyme properties are sufficiently distinct to warrant separate categorization. Accordingly, it is useful to classify ribonucleotide reductase types on the basis of 1) cofactor requirements and 2) the observed occurrence of negative allosteric effectors.

#### Manganese Requiring Ribonucleotide Reductases

In certain gram positive bacteria, manganese deficiency has been shown to produce unbalanced growth, filamentous morphology, and the arrest of DNA but not RNA or protein synthesis (Webley et al., 1962; Oka et al., 1968). These effects had been attributed to manganese-controlled DNA precursor biosynthesis (Auling and Follmann, 1980). A manganese requiring ribonucleotide reductase has recently been reported to occur in the coryneform bacteria, Brevibacterium ammoniagenes and Micrococcus luteus (Schimpff-Weiland et al., 1981). The apoenzyme, reactivated with  $\text{Fe}^{2+}$  ion, was only 50% as active than

TABLE I

Properties of Different Classes of Ribonucleotide Reductase

Class	Source	Required Cofactor	Substrate	#Subunit Types	Subunit Size (M <sub>r</sub> )	Allosteric Control		Ref.
						(effector)	(substrate)	
1.	<u>Micrococcus luteus</u>	Mn <sup>++</sup>	NTP or NDP	-	-	dTTP + dATP +	GTP, GDP CTP, CDP	Shimpff-Weiland et al., 1981
2.	<u>Lactobacillus leichmannii</u>	5'-deoxy-5'-adenosylcobalamin (AdoCbl)	NTP	1	76,000	dATP + dCTP + dTTP + dGTP +	CTP UTP GTP ATP	Goulian and Beck, 1966
	<u>Anabaena</u> sp.	AdoCbl + Ca <sup>++</sup>	NTP	1	72,000	dATP + dTTP + dGTP +	CTP GTP ATP	Gleason and Frick, 1980
(UTP reduction not observed)								
3.	<u>Cornybacterium nephridii</u>	AdoCbl	NDP	1	100,000 (dimer)	dATP, dTTP - dGTP +	CDP, GDP ADP, UDP	Tsai and Hogenkamp, 1981
4.	<u>E. coli</u>	Fe <sup>II</sup>	NDP	2	84,000 (B1) 39,000 (B2)	Complex		Thelander and Reichard, 1979
	calf thymus	Fe <sup>II</sup>	NDP	2	84,000 (M1) 58,000 (M2)	Complex		ibid.

when incubated with the preferred  $Mn^{2+}$  cofactor. In preparations which were not  $Mn^{2+}$  depleted, exogenous  $Mg^{2+}$  stimulated enzymatic activity to the same degree as added  $Mn^{2+}$ . This suggested that  $Mg^{2+}$  ion may exhibit a second type of interaction with the ribonucleotide reductase. Deoxyadenosylcobalamin did not stimulate the enzymatic activity. The guanosine nucleotides were the best substrates (GTP;  $K_m = 2 \times 10^{-5}$ , at pH 7.5) and the enzyme fraction was capable of reducing both GDP and GTP at comparable rates. However, this dual specificity may have been due to contaminating nucleotide kinases or phosphatases in the cellular extract. The existence of negative allosteric effectors was not reported.

#### Adenosylcobalamin Requiring Ribonucleotide Reductases

The second category of ribonucleotide reductases are characterized by an absolute requirement for a 5'-deoxy-5'-adenosylcobalamin cofactor. These vitamin  $B_{12}$ -dependent ribonucleotide reductases (E.C. 1.17.4.2) catalyze the reduction of ribonucleoside di- or triphosphates. The most well characterized example of an enzyme in this group is the ribonucleotide reductase from Lactobacillus leichmannii. The requirement of vitamin  $B_{12}$  for deoxyribonucleotide biosynthesis was first demonstrated using crude L. leichmannii extracts and CTP as substrate (Blakely and Barker, 1964). Ribonucleotide reductase from this source was subsequently purified to homogeneity and a detailed study of its physical nature and reaction mechanism undertaken (Goulian and Beck, 1966). This preparation was shown to be capable of reducing all four ribonucleoside triphosphates.

## Ribonucleotide Reductase from *L. leichmannii*

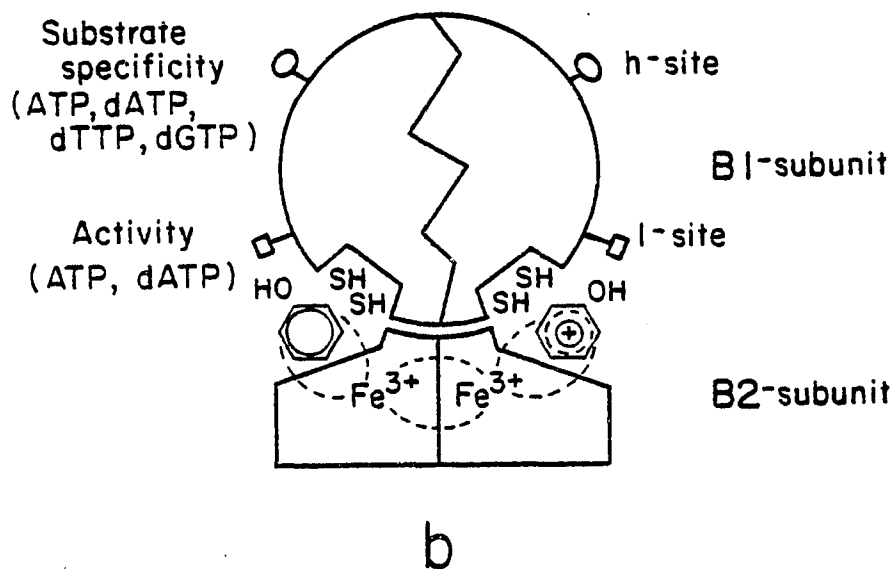
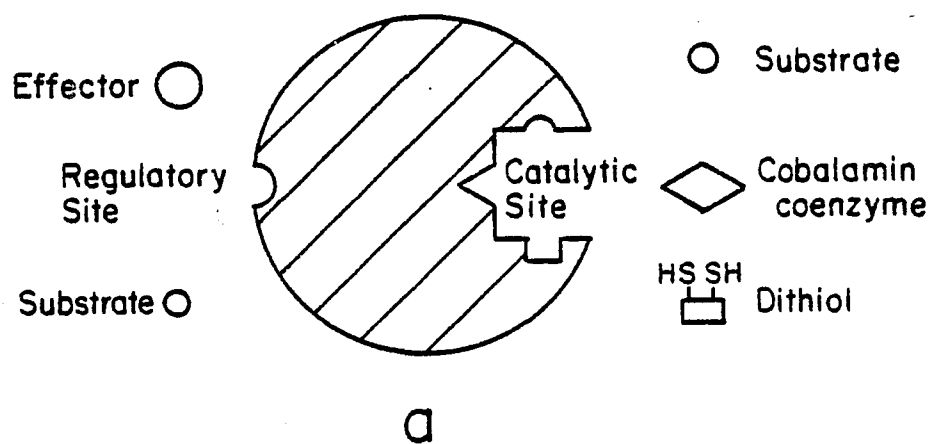
### Structural Features

The enzyme is a monomer with a molecular weight of 76,000 and  $s_{20,W}$  of 5.3 (Panagou et al., 1972; Chen et al., 1974). Available structural information on the protein is summarized in Figure 2 (Thelander and Reichard, 1979). A common binding site for positive allosteric effector deoxyribonucleoside triphosphates has been demonstrated with  $K_d$ 's ranging from 9  $\mu$ M to 80  $\mu$ M (Chen et al., 1974; Singh et al., 1977). Ribonucleoside triphosphates also interact with this regulatory site but with an affinity several orders of magnitude lower. Demonstration of a substrate binding site is not possible by equilibrium dialysis technique because kinetic measurements have shown that the apparent  $K_m$  for GTP is as high as 0.24 mM. However, deoxyribonucleotides display little product inhibition, an observation consistent with the existence of two separate sites. That coenzyme binding requires effector binding to the regulatory site has been directly demonstrated (Singh et al., 1977). Although thioredoxin binding has not been demonstrated, interaction with the catalytic site is assumed.

### Reaction Mechanism

The majority of coenzyme  $B_{12}$  reactions involve the adenosylcobalamin moiety as an intermediate hydrogen carrier which abstracts a specific hydrogen from the substrate and returns a hydrogen to a different position in the product (Abeles and Dolphin, 1976; Abeles, 1971). This type of enzyme, accordingly, would catalyze the transfer

Figure 2. Models of (A) ribonucleoside triphosphate reductase from Lactobacillus leichmannii and (B) ribonucleoside diphosphate reductase from Eschericia coli (from Thelander and Reichard, 1979).



of  $^3\text{H}$  from  $[5'\text{-}^3\text{H}]\text{-adenosylcobalamin}$  to product.

The catalytic mechanism for the ribonucleotide reductase involves the stereospecific reduction of ribonucleoside triphosphate. The 2'-hydrogen of the deoxyribose product is uniquely derived from the solvent. Hence, this reaction is different from that catalyzed by other  $\text{B}_{12}$ -dependent enzymes. Incubation of  $[5'\text{-}^3\text{H}]\text{-adenosylcobalamin}$  with the ribonucleotide reductase does not result in transfer of  $^3\text{H}$  to deoxyribose, but complete  $^3\text{H}$  exchange with the solvent (Abeles and Beck, 1967; Hogenkamp *et al.*, 1968).

This transfer requires a dithiol (thioredoxin, dithioerythritol, etc.) which is the proposed carrier during the hydrogen exchange. Both adenosylcobalamin 5'-methylene hydrogens are involved in the transfer, although they are stereochemically non-equivalent. This led to the hypothesis that an enzyme bound intermediate containing three equivalent hydrogens is formed, one derived from the reducing thiol (Hogenkamp *et al.*, 1968). Evidence for the formation of such an intermediate has been obtained experimentally by EPR measurements (Orme-Johnson *et al.*, 1974) and spectrophotometric stopped-flow studies (Tamao and Blakey, 1973). In the presence of allosteric effector (dGTP) and dithiol, adenosylcobalamin reacts rapidly ( $t_{1/2}=17$  ms) with equimolar amounts of reductase. Spectral changes in the UV and visible regions were observed which correspond to those predicted for partial homolytic cleavage of the coenzyme carbon-cobalt bond to yield cob(II)alamin and a 5'-deoxyadenosyl radical. Also, at  $-130^\circ\text{C}$ , the reaction mixture has a characteristic EPR spectrum indicating the pro-

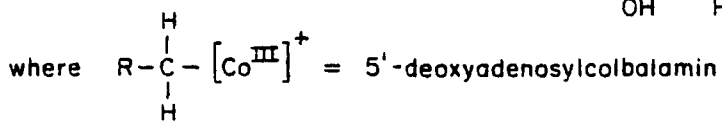


duction of two unpaired electrons per molecule of intermediate. The EPR signal is centered at  $g = 2.119$ . Spectrum hyperfine structure analysis by computer simulation indicates that the principle signal features are due to an unpaired electron associated with  $\text{Co}^{2+}$  and an underlying broadened signal attributed to the 5'-deoxyadenosyl radical (Walker et al., 1974).

A second paramagnetic species identified as cob(II)alamin is formed slowly ( $t_{1/2} = 30$  min) by the enzyme in the presence of dithiol and effector. This species is considered to be a side reaction product (Sando et al., 1974; Yamada et al., 1971). In addition, a third type of EPR signal is observed in the presence of the complete ribonucleotide reductase system: enzyme, cofactor  $\text{B}_{12}$ , ribonucleoside triphosphate substrate, allosteric effector, and dithiol. The signal, termed the "doublet" EPR spectrum consists of two major lines with mean  $g$  values of 2.032 and 1.965 (Hamilton et al., 1972). The possible function of this species as an intermediate is not ruled out by its slow formation ( $T_{1/2} \sim 10$  min). By analogy with another  $\text{B}_{12}$ -dependent enzyme, dioldehydrase, which also exhibits a doublet EPR signal (Valinsky et al., 1964), the species has been attributed to a radical derived from the reducing substrate or from the ribonucleotide substrate (Hogenkamp and Sando, 1974).

This information suggests the presence of at least two intermediates in the L. leichmanni ribonucleotide reductase catalyzed reaction. A reaction sequence consistent with this information has been proposed (Hogenkamp and Sando, 1974) (Figure 3). The formation of cob(II)-

Figure 3. Proposed reaction mechanism for B<sub>12</sub>-dependent ribonucleotide reductase (Hogankamp & Sando, 1974).



T = enzyme or reducing cofactor

alamin and a 5'-deoxyadenosyl radical (species I) is a result of enzyme catalyzed homolytic cleavage of the carbon-cobalt bond in the 5'-deoxyadenosylcobalamin coenzyme. This 5'-deoxyadenosyl radical then abstracts a hydrogen from a dithiol, provided by either reducing substrate or enzyme, to yield a sulfur radical (species II) and 5'-deoxyadenosine. Since both of these steps are reversible, the hydrogen isotope exchange discussed previously is possible. The sulfur radical is then postulated to abstract a hydroxyl radical from the 2'-carbon of the nucleotide substrate, resulting in the formation of a 2'-deoxyribonucleotide radical (III), water, and a disulfide. Finally, coenzyme radical pair regeneration occurs as species III abstracts a hydrogen atom from 5'-deoxyadenosine to yield the 2'-deoxyribonucleotide product. The "doublet" EPR spectrum is attributed to either the 2'-deoxyribonucleotide radical or the sulfur radical interacting with the unpaired electron of cob(II)alamin (Babior et al., 1974).

Recently, additional mechanistic information has become available which suggests that the actual reaction scheme may be more complex. In double label experiments using  $[3'\text{-}^3\text{H}]\text{-UTP}$  and  $[^{14}\text{C}]\text{-UTP}$  as ribonucleotide reductase substrates, a decrease in the specific activity ratio  $[^3\text{H}/^{14}\text{C}]$  was observed in the formation of dUTP product (Stubbe et al., 1981). The selection effect against  $^3\text{H}$  was 1.8, a result which suggests cleavage of the 3'-carbon-hydrogen bond of  $[3'\text{-}^3\text{H}]\text{-UTP}$  during substrate conversion to 2'-dUTP.  $^3\text{H}$  was neither released to solvent nor recovered in re-isolated  $\text{B}_{12}$  coenzyme. Thus, the adenosylcobalamin cofactor may not directly abstract from the 2' position of substrate. These results are consistent with a reaction mechanism

involving the formation of a thiol radical (II) and deoxyadenosine. A protein-associated radical species is proposed to interact with the 3'-H of the substrate and not abstract a hydroxyl radical from the 2' carbon as indicated (Stubbe et al., 1981). The implications of 3'-H bond lability, an effect also observed in the E. coli ribonucleotide reductase system (Stubbe and Ackeles, 1980), upon the proposed mechanism for the enzymatic reduction of ribonucleotides will be discussed in the E. coli ribonucleotide reductase mechanism section.

### Allosteric Regulation

Allosteric regulation of substrate specificity is a feature of the L. leichmannii enzyme. The most important result of effector binding to the regulatory site is an increase in the protein's affinity for coenzyme (decrease in  $K_d$  from 0.93 to 0.2 mM with 2 mM dGTP at 5°C). Enhancement of cofactor binding shows little specificity towards a particular deoxyribonucleotide. The identity of the allosteric effector plays a significant part in dictating the substrate specificity. Table I lists the most positive allosteric effectors for the enzyme. A strong negative allosteric effector has not been observed (Thelander and Reichard, 1979). Allosteric control effects are presumed in many instances to involve enzyme subunit interactions, e.g., cooperative effects. However, the monomeric L. leichmannii enzyme does not associate to form higher oligomers. Instead, the ribonucleotide reductase exhibits significant changes in tertiary structure upon effector binding (Singh et al., 1977; Kim et al., 1977).

A second example of an enzyme from this group is the ribonucleo-

tidal reductase from the blue-green alga Anabaena 7119 sp. (Gleason and Frick, 1980). The purified protein displays properties similar to those previously described. The unique feature of this enzyme is that calcium ion preferentially stimulates activity to twice the degree of that observed with magnesium (the usual metal cofactor).

A survey of different organisms has evaluated the distribution of B<sub>12</sub>-dependent reductases. This enzyme type has been reported to be more common among prokaryotes than the iron-requiring type (Class 4). Also, among the eukaryotic organisms surveyed only Euglenophyta and the fungus Phytomyces chartarum exhibited adenosylcobalamin-dependent ribonucleotide reductase activity. However, a general convention regarding enzyme type and source has not yet been established (Gleason and Hogenkamp, 1972).

#### Ribonucleotide Reductase from *C. nephridii*

A ribonucleotide reductase from Corynebacterium nephridii, which also requires a B<sub>12</sub> cofactor has been reported (Tsai and Hogenkamp, 1980). This enzyme has been grouped separately since it displays several distinctive physical properties which indicate it to be a more complex protein than those previously discussed. This ribonucleotide reductase catalyzes the reduction of the four common ribonucleoside diphosphates and hydrogen exchange between adenosylcobalamin and the solvent. In contrast, ribonucleoside triphosphates are the preferred substrate for the majority of other B<sub>12</sub>-requiring enzymes. The reductase activity is modulated by both positive (dGTP) and negative (dATP, dTTP) allosteric effectors. Negative allosteric control has not been described for other B<sub>12</sub>-dependent reductases. This type of

control, in concert with positive effectors, allows for a greater degree of activity regulation. Furthermore, the C. nephridii enzyme behaves as a dimer with a subunit molecular weight of 100,000. Cooperativity, implied by the oligomeric enzyme structure, represents a later development and evolutionary advantage (Koshland, 1976). Hence, the C. nephridii reductase oligomeric structure, advanced allosteric control capability, and capacity to reduce ribonucleoside diphosphates suggests the enzyme to be an intermediate between that enzyme type from L. leichmannii and the more complex iron-requiring reductase systems. C. nephridii, a facultative anaerobe, is known to be more advanced in evolutionary terms than the microaerophile, L. leichmannii. (Tsai and Hogenkamp, 1980). The complex nature of the C. nephridii ribonucleotide reductase may be a reflection of this evolutionary relationship.

#### Iron-Requiring Ribonucleotide Reductases

The final category of ribonucleotide reductase types is comprised of those enzymes which characteristically require protein-associated iron for activity instead of an adenosylcobalamin coenzyme. Among the prokaryotes, this enzyme class has been most extensively studied utilizing the enzyme from E. coli (Thelander and Reichard, 1979). The E. coli ribonucleotide reductase serves as a convenient prototype for the other enzymes in this group, most notably those from mammalian sources. Other iron-requiring reductases have been identified from bacteriophage T<sub>4</sub>-infected E. coli cells (Berglund, 1975), the cyanobacterium Agmenellum quadruplicatum (Gleason and Wood, 1976), the alga Scenedesmus obliquus and Chlorella pyrenoidosa (Feller and Follman, 1976), wheat germ (Müller et al., 1973), and the silkworm

Hyalophora cecropia (Swindlehurst et al., 1971). For those determinations made in mammalian tissues, the ribonucleotide reductase activity has consistently been among this grouping (Thelander and Reichard, 1979).

#### Ribonucleotide Reductase from *E. coli*

Ribonucleotide reductase from *E. coli* (E.C. 1.17.4.1) is composed of two non-identical subunits designated proteins B1 and B2 (Brown et al., 1967). Two closely linked structural genes *nrdA* and *nrdB*, which are situated at 48 min (between *nalA* and *glpT*) on the *E. coli* genome (Bachman et al., 1976), code for the complementary subunits. A coordinate regulation of these two genes, apparently located within the same operon, has been observed. Operon transcription results in the production of a polycistronic mRNA precursor, transcribed in the direction *nrdA* to *nrdB* (Hanke and Fuchs, 1982).

#### Structural Features

A model for the quaternary structure of the *E. coli* ribonucleotide reductase is shown in Figure 2. The active enzyme is an oligomer of the regulatory (B1) and catalytic (B2) subunits, each a dimeric species in 1:1 stoichiometry, forming an active tetramer. Formation of the active complex by the complementary subunits, which separately show no catalytic activity, is dependent upon the presence of magnesium ions (Brown et al., 1967).

#### B1 Subunit

The dimeric regulatory subunit (B1) has a molecular weight of 160,000 (Brown et al., 1969b). End group analysis indicates the presence of identical -COOH termini for the polypeptide chains which are



of similar or identical size. However, a difference in  $-NH_2$  termini for each polypeptide has been observed (Thelander, 1973), suggesting an  $\alpha$  ' structure. This difference does not appear to necessarily reflect the existence of two separate structural genes, since it may be an artifact of purification (Thelander and Reichard, 1979). Each polypeptide is assumed to have an identical function. The protein B1 dimer can be dissociated by removal of magnesium ions or upon oxidation of its cysteine residues. Protein B1 dissociation results in the concomitant loss of enzymatic activity when assayed in the presence of complementary B2 subunit. Incubation of the B1 subunit with dithiothreitol is effective in reversing the dissociation/inactivation process (Thelander, 1973).

The B1 subunit contains binding sites for both the ribonucleoside diphosphate substrates (von Döbeln and Reichard, 1976) and the allosteric effector nucleoside triphosphates (Brown and Reichard, 1969b). Two substrate-binding sites have been demonstrated on each protein B1 dimer by equilibrium dialysis technique (von Döbeln and Reichard, 1976). Either of these apparently identical sites can bind any one of the four ribonucleoside diphosphate substrates. Substrate specificity and binding affinity are determined, however, by the identity of allosteric effector(s) which bind to distinct regulatory sites. Effector binding dictates the conformation of the substrate binding site and thus determines substrate specificity.

The B1 molecule has been shown to contain two types of effector binding sites (l or h) each type consisting of two subsites (per

dimer) (Brown and Reichard, 1969b). The affinity of each effector site for dATP is the variable utilized for the classification of effector site type. h-sites have a high affinity for dATP ( $K_d = 3 \times 10^{-8}$  M), whereas l-sites exhibit a decreased dATP affinity ( $K_d = 1-5 \times 10^{-7}$  M). Competition experiments have shown that in addition to dATP, l-sites bind only ATP, but h-type sites also bind ATP, dTTP, and dGTP.

### Allosteric Regulation

Overall enzyme activity is determined by the l-site since binding of ATP or dATP results in active or inactive ribonucleotide reductase, respectively. Hence, the ratio of adenine nucleoside triphosphate pools determines overall enzyme activity, in vivo. Substrate specificity is controlled by the identity of allosteric effector bound to the h-site, when ATP is bound to the l-site (activity). This event influences both the  $K_m$  and  $V_{max}$  during catalysis for a given substrate (Thelander and Reichard, 1979). A compilation of the influences of various effectors upon enzyme activity is presented in Table II.

Allosteric effectors also induce changes in the sedimentation behavior of the enzyme. Proteins B1 (7.8s) and B2 (5.8s) combine in the presence of magnesium ion to form an active complex (9.7s). dATP causes a shift in the protein B1B2 complex sedimentation rate from 9.7s to 15.5s. The larger species, which contains equimolar subunit amounts, represents an inactive form of the enzyme and is believed to be a dimer of the 9.7s complex (Brown and Reichard, 1969a). This type of behavior is consistent with the regulatory control effects observed in other allosteric enzyme systems.

TABLE II

Allosteric Regulation of  
Ribonucleotide Reductase from E. coli<sup>a</sup>  
(from Thelander and Reichard, 1979)

Effector binding to		Reduction of			
1-sites	h-sites	CDP	UDP	GDP	ADP
0	ATP	+	+	0	0
0	dTTP	+	+	+	+
0	dGTP	0	0	+	+
ATP	ATP or dATP	+	+	0	0
ATP	dTTP	-	-	+	(+)
ATP	dGTP	nd	nd	(+)	+
dATP	any effector	-	-	-	-

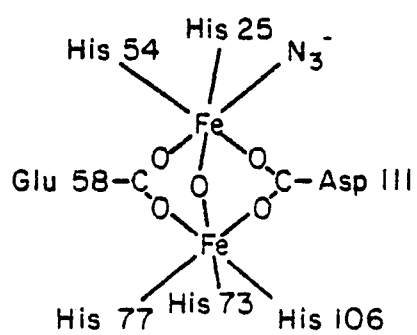
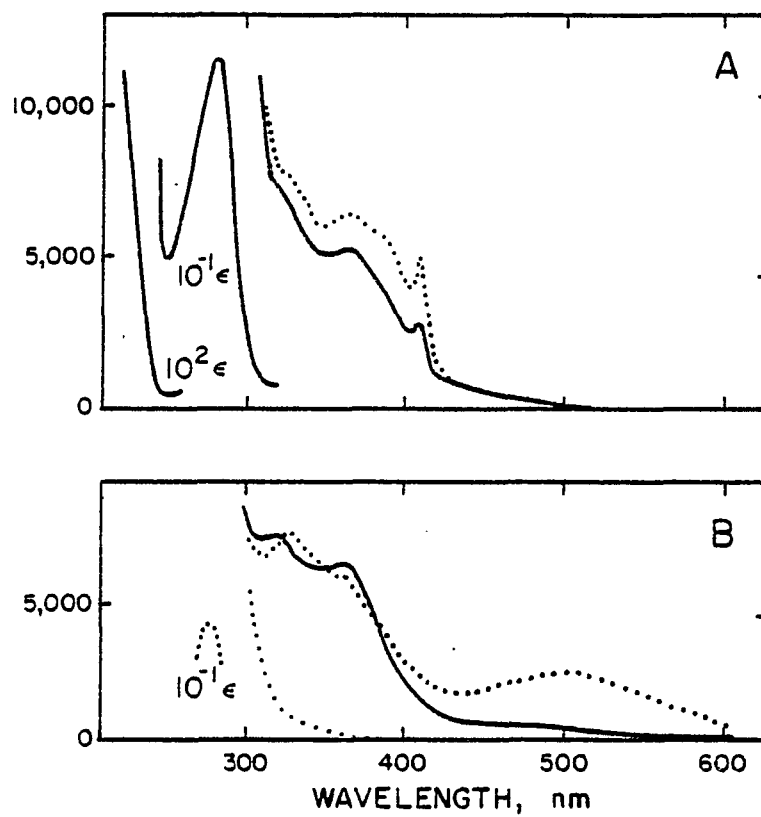
<sup>a</sup>0 = no effect; + = stimulation; - = inhibition;  
and nd = not determined.

The diagram shown in Figure 2 arbitrarily assumes effector binding site symmetry within the dimeric B1 subunit, consistent with a reasonable structural model. However, an asymmetric distribution of effector binding cannot be ruled out and may be related to the possible  $\alpha$  ' protein B1 structure (Thelander and Reichard, 1979).

### B2 Subunit

The B2 (catalytic) subunit of E. coli ribonucleotide reductase is a dimer of molecular weight 78,000 and consists of two identical polypeptides. The subunit contains two atoms of non-heme iron; one per chain is assumed (Brown et al., 1969a). The necessity of protein B2-associated iron for enzymatic activity has been well documented (Thelander and Reichard, 1979). Iron removal by subunit dialysis against 8-hydroxyquinoline or EDTA results in a loss of enzymatic activity, although apoprotein B2 can still form a 1:1 complex with protein B1. The intact apoprotein can be reactivated to greater than 100% of its original activity with various iron salts, e.g., Fe(II)ascorbate. The subunit iron is inert to reducing and oxidizing agents, is not bonded to inorganic sulfur, and can be released by cold acid treatment (Brown et al., 1969a; Thelander and Reichard, 1979). The exact nature of the protein B2 iron center has been carefully evaluated. The electronic (Figure 4)(Ehrenberg and Reichard, 1972) and Mossbauer spectra (Atkin et al., 1973) are very similar to those for oxy- or methydroxo-hemerythrin (Klotz et al., 1976; Stenkamp et al., 1976). The structure of the binuclear iron center in the Fe(III) form of the oxygen transport protein hemerythrin has been elucidated by x-ray crystallographic (Figure 4)(Stenkamp et al., 1981; Hendrickson, 1981) and x-ray absorption spectroscopic analysis (Hendrickson et al., 1982). The data

Figure 4. Electronic spectra of (A) active protein B2, native (——) and reconstituted (.....); (B) hemerythrins: methydroxohemerythrin (——), oxyhemerythrin (.....), and deoxy- and metal-free hemerythrins (—.—) (Atkin et al., 1973), (C) the iron complex in metazidohemerythrin from Themiste zostericola (Sheriff et al., 1982).



indicates the presence of two iron atoms which are coordinated to protein imidazoles and are bridged by two protein carboxylates and an oxo group from the solvent (Sheriff et al., 1982). The strong antiferromagnetic interaction between the ferric ions and the intense electronic absorption bands in the near-UV spectra of both met- and oxyhemerythrins are attributed to the  $\mu$ -oxo bridge (Garbett et al., 1969).

The protein B2 Mossbauer data suggested two possible structures for the iron center(s) in the protein molecule: a) two low spin Fe(II) complexes in separate sites or b) one complex of two antiferromagnetically coupled iron atoms, both in the Fe(III) high spin state (Atkin et al., 1973). By analogy with the active center data for hemerythrin, both the electronic and Mossbauer data for protein B2 favored the latter (b) case.

The paramagnetic susceptibility of protein B2 has been measured over the temperature range 30-200 K (Petersson et al., 1980). A deviation from the Curie law was observed at high temperatures which is consistent with the occurrence of an antiferromagnetically coupled pair of high spin Fe(III) ions. Also, an exchange coupling of  $-J = 108 \text{ cm}^{-1}$  was calculated. The magnitude of this value is similar to those  $-J$  values previously reported for a number of  $\mu$ -oxo bridged high spin ferric ion model complexes (Murray, 1974), oxyhemerythrin ( $77 \text{ cm}^{-1}$ ), and metaquo-hemerythrin ( $134 \text{ cm}^{-1}$ ) (Dawson et al., 1972). The magnitude of exchange interaction is thought to be a property of the  $\text{Fe}^{3+}-\text{O}^{2-}-\text{Fe}^{3+}$  bridge, and not appreciably related to iron ligand

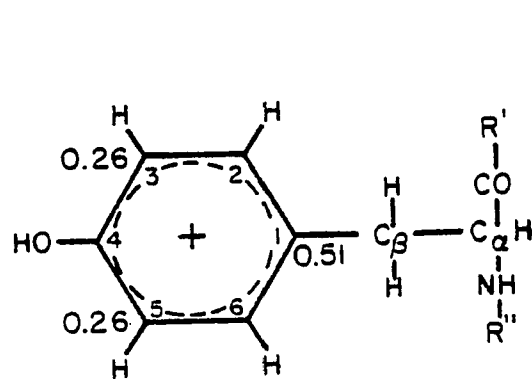
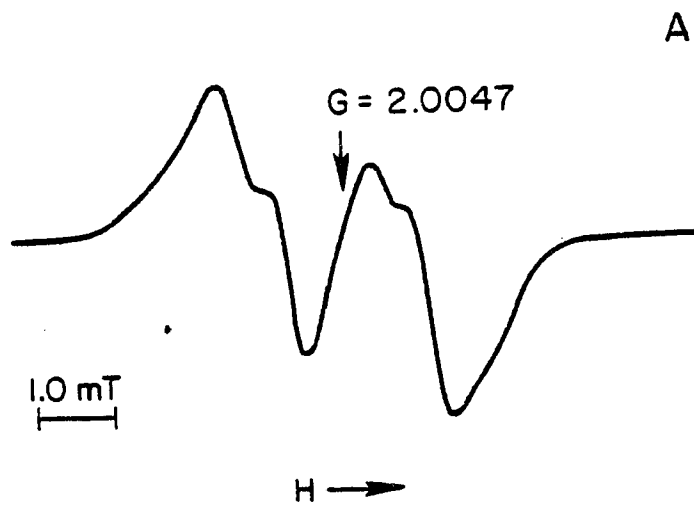
groups (Murray, 1974). Thus, a u-oxo bridged iron dimer is indicated in the B2 subunit of ribonucleotide reductase.

Additional evidence for the existence of an antiferromagnetically coupled binuclear iron center in protein B2 has been obtained using Resonance Raman spectroscopy. Raman spectra of both native and hydroxyurea treated protein B2 exhibit a resonance enhanced Raman band at  $496\text{ cm}^{-1}$  (Sjöberg et al., 1980) in resonance with the 370 nm electronic transition of the binuclear iron center (Sjöberg et al., 1982). This transition has been assigned to an Fe-O vibrational mode arising from an oxygen-containing ligand. Tyrosinate ring Raman modes were not observed, diminishing the possibility that protein B2 is an iron-tyrosinate protein. In the presence of  $\text{H}_2^{18}\text{O}$ , the peak shifts to  $481\text{ cm}^{-1}$ , an effect indicative of solvent oxygen exchange with the Fe-O moiety. The measured rate of exchange ( $K = 8.3 \times 10^{-4}\text{ s}^{-1}$ ) was consistent with a u-oxo bridged structure. In addition,  $^2\text{H}_2\text{O}$  failed to shift the Fe-O vibration to a lower frequency, suggesting that protonation of the oxygen was unlikely (Sjöberg et al., 1980, 1982).

The B2 subunit of the ribonucleotide reductase also contains a free radical characterized by a doublet EPR signal (Figure 5) centered around  $g = 2.0047$  (Ehrenberg and Reichard, 1972). This moiety is distinct from the iron center, which both magnetic susceptibility and Mossbauer spectroscopic experiments have established as being diamagnetic. Isotope substitution experiments have resulted in the assignment of the radical to a tyrosine residue in the protein (Sjöberg et al., 1977). The main feature of the EPR spectrum is a doublet split-



Figure 5. (A) Electronic spin resonance spectrum of protein B2 at 77K (Sjöberg and Graslund, 1978); (B) the tyrosyl free radical of protein B2 with estimated spin densities at C-1, C-3 and C-5 (Sjöberg et al., 1978).



ting of about 1.9 mT. This feature arises from radical hyperfine coupling to one of the  $\beta$  protons of tyrosine. Isotropic hyperfine coupling of the radical to both  $\beta$ -hydrogen atoms of the tyrosyl methylene carbon, in which case the major spectral feature would be a triplet splitting, is not observed. Hyperfine coupling of a proton bound to a carbon atom neighboring a carbon atom with spin density is dependent upon radical geometry (Stone and Maki, 1962). If the C- $\beta$  H- $\beta$  bond is near the plane of the aromatic ring, then hyperfine coupling of an electron occupying the  $P_z$  orbital of  $C_1$  of the aromatic ring to an adjacent methylene proton ( $0 \sim 90^\circ$ ) would be negligible. Therefore, the observed doublet splitting is attributed to a locked steric configuration of the tyrosyl residue (Sjöberg et al., 1978). The incorporation of [ $\beta$ - $^{13}\text{C}$ ] tyrosine into protein B2 results in a slight broadening (0.5 mT) of the signal, an effect consistent with the  $^{13}\text{C}$  nucleus position neighboring a position with spin density. Also, a partially resolved triplet splitting of about 0.7 mT is observed with the relative intensities of 1:2:1 (Sjöberg et al., 1977). Incorporation of [3,5- $^2\text{H}_2$ ] tyrosine into protein B2 results in a loss of this hyperfine structure. This effect was not observed in substitution experiments using [2,6,  $\alpha$ - $^2\text{H}_3$ ] tyrosine. Thus, significant hyperfine coupling of the unpaired electron has been assigned to its interaction with H-3 and H-5 tyrosyl nuclei. Based upon these findings, a free radical model has been proposed in which the unpaired electron resides in the aromatic ring of tyrosine (Figure 4b). Spin density on C-1 gives rise to hyperfine coupling to one of the  $\beta$ -hydrogen atoms and spin density on C-3 and C-5 give rise to anisotropic hyperfine couplings with H-3 and H-5, which result in the

partially resolved 1:2:1 triplet splitting (Sjöberg et al., 1978).

The tyrosyl free radical of protein B2 is believed to be a cationic species presumably formed by an iron catalyzed 1-electron aerobic oxidation of the tyrosine residue (Thelander and Reichard, 1979). The EPR spectral properties of oxidized tyrosine, formed by the loss of an electron from the aromatic ring (Box et al., 1974) closely resemble that observed in the E. coli ribonucleotide reductase system (Sjöberg et al., 1978). Formation of the free radical is closely linked to the presence of iron in subunit B2. Iron removal results in radical loss. Radical regeneration can be achieved, however, by apoprotein reconstitution. Selective radical destruction, without loss of iron, occurs during protein B2 aging or by chemical treatment with radical scavenging agents, e.g., hydroxyurea or hydroxylamine. These do not alter the properties of the binuclear iron center (Atkin et al., 1973). Enzymatic activity and the radical presence are regained only when the iron is removed from protein B2 which is then reconstituted with  $\text{Fe}^{2+}$  iron in the presence of  $\text{O}_2$  (Petersson et al., 1980). The reconstitution step must be a complex set of events; the oxidation of  $\text{Fe}^{2+}$  to the high spin Fe(III) complex apparently occurs in situ, and results in the generation of a tyrosyl radical within each protein B2 molecule. Binding of molecular oxygen to a binuclear iron center during proposed u-oxo bridge formation could activate it for the abstraction of an electron from a neighboring tyrosine residue (Sjöberg et al., 1982).

Both the protein B2 free radical and iron center contribute to

the characteristic UV-visible absorption spectrum (Figure 3a) of the subunit. Characteristic protein B2 absorption maximum are located at 280, 370, 410, 500 and 600 nm. A steep shoulder is observed at 325 nm (Brown et al., 1969a; Petersson et al., 1980). Removal of iron from the subunit results in the disappearance of the absorption maximum, with the exception of the 280 nm peak and the shoulder at 325 nm. Apoprotein reconstitution regenerates the spectrum.

Selective destruction of the protein B2 radical by hydroxyurea treatment does not cause significant changes in the iron center, but does cause a loss of the shoulder at 390 nm, the sharp 410 nm peak, and a height reduction of the 600 nm peak. Hence, the electronic spectrum of hydroxyurea-treated protein B2 above 300 nm has been assigned to the iron center (Atkin et al., 1973). This spectrum is analogous to those previously reported for oxyhemerythrin and several methemerythrins (Klotz, 1971).

A difference spectrum between native and radical-free protein B2 has been generated (Petersson et al., 1980). Obvious similarities exist between this difference spectrum (tyrosyl free radical of protein B2) and those reported for radicals of tyrosine and tritertiary butyl phenol (Feitelson and Hayon, 1973; Land et al., 1961). Thus, the electronic spectrum features of the B2 subunit can be assigned to separate components generated by the radical and by the binuclear iron center (Petersson et al., 1980).

#### Active Site

Structural elements for the active site of the ribonucleotide

reductase are contributed by both the B1 (binds substrate and effectors) and B2 (tyrosyl free radical containing) subunits. Evidence for this has been obtained by enzyme inhibition studies using substrate analogues (Thelander et al., 1976). 2'-deoxy-2'-chloro ribonucleoside diphosphate and 2'-deoxy-2'-azido ribonucleoside diphosphate irreversibly inactivate the B1 and B2 subunits respectively, without effecting the complementary subunit in either case. The inhibition is substrate specific since it requires the presence of both complementary subunits, is controlled by allosteric effectors, and can be competitively protected against by normal substrates.

Protein B1 inactivation is caused by modification of subunit dithiols which participate in the oxidation-reduction reaction. As a consequence of enzyme interaction, the chloro-inhibitor decomposes into free base, chloride ion, and 2'-deoxyribose-5'-diphosphate (Thelander et al., 1976). Analogous results have also been reported for the 2'-fluoro derivative (Stubbe and Kozarick, 1980). Conversely, the azido derivative functions as a radical scavenger, and upon reduction by ribonucleotide reductase, selectively destroys the free radical of B2. This information indicates an enzyme active site formed by both subunits, in which the electron-donating oxidation-reduction active dithiols of B1 are in close proximity to the free radical of B2 (Thelander et al., 1976).

The essential role of oxidation-reduction active sulfhydryls contributed by the B1 subunit (21 half-cystine residues/mol B1) in the catalytic mechanism of the E. coli ribonucleotide reductase reaction

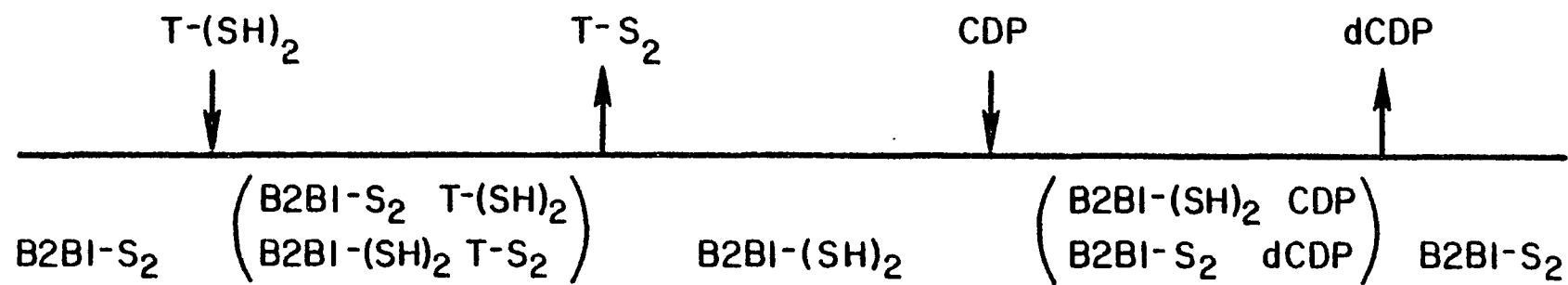
has been demonstrated by kinetic analysis in the absence of a hydrogen donor system. Under these conditions, ribonucleotide reductase converts a maximum of 3 moles of substrate to product per mole of protein B1, while 6 moles of protein B1 sulfhydryls are oxidized. The reaction requires the presence of protein B2 (10 half-cystine residues/mol protein B2), but oxidation-reduction-active sulfhydryls from this subunit could not be detected. Also, electron transfer was shown to occur readily between fully reduced B1 and the oxidation-reduction-active disulfide of thioredoxin (Thelander, 1974). Steady state kinetic analysis indicates a ping-pong mechanism, e.g., two stable enzyme forms during catalysis (Figure 6). During ribonucleotide reduction, electron flow is proposed to be from thioredoxin to the disulfides residing on protein B1. These protein associated dithiols would then interact with the protein B2 free-radical to reduce the ribonucleotide (Thelander, 1974).

#### Hydrogen Transport System

The requirement for a hydrogen transport system in the reduction of ribonucleotides led to the discovery of the thioredoxin system in E. coli (Laurent et al., 1964) and eukaryotes (Moore, 1967). In addition to thioredoxin, the FAD-containing thioredoxin reductases appear to be ubiquitous. E. coli thioredoxin reductase occurs as a dimer  $[(FAD)_2 \alpha_2]$  with a subunit molecular weight of 66,000. Each polypeptide chain contains one oxidation-reduction-active disulfide (Thelander, 1968) which is reduced by  $FADH_2$ , and in turn reduces the B1 associated half-cystine residues. Thus, a shuttle of  $S_2/(SH)_2$  interchanges mediates the transfer of electrons from NADPH to ribonucleotides (Thelander, 1974). The physiological role of thioredoxin

Figure 6. Cleland notation for the reaction catalyzed by the ribonucleotide reductase system.  $B_2B_1-S_2$  and  $B_2B_1-1(SH)_2$ , oxidized and reduced form of protein B1, in the ribonucleotide reductase complex;  $T-S_2$  and  $T-(SH)_2$ , oxidized and reduced form of thioredoxin. (Thelander, 1974).





is to participate in thiol-dependent oxidoreductions (Holmgren, 1981; Holmgren and Fagerstedt, 1982). Mutants of E. coli, lacking in either thioredoxin (Mark and Richardson, 1976) or thioredoxin reductase (Fuchs, 1977), have been observed which show no decreased ability to reduce ribonucleotides. An alternate glutaredoxin, glutaredoxin reductase system has been discovered in E. coli mutants lacking thior- edoxin system components (Holmgren, 1976) and also in wild type E. coli (Thelander and Reichard, 1979). The glutaredoxin hydrogen transport system is highly analogous to its thioredoxin utilizing counterpart except that the protein is reduced directly by glutathione. This step constitutes a unique disulfide reduction by a monothiol compound. The role of either protein as a preferred or obligatory cofactor in ribonucleotide reduction is not as yet well defined.

#### Reaction Mechanism

As in the L. leichmannii enzyme system, the mechanism of ribonucleotide reduction has been the subject of careful study. The selective reduction of one of the two secondary hydroxyl groups of ribose to a methylene, with retention of configuration at neutral pH and physiological temperature is proposed to occur by direct reduction, without the occurrence of stable intermediate products. Reaction sequences, e.g., dehydration-hydrogenation, 2'-OH oxidation-reduction, and 2'-OH activation by phosphorylation, involving such intermediates have been ruled out by isotope, chemical, and NMR-spectroscopic analysis (Follmann, 1974).

The interaction of E. coli ribonucleotide reductase with 2'-chloro or 2'-fluoro nucleoside diphosphates has been described above.

The 2'-fluoro substrate analogue reaction also results in cleavage of the N-glycosyl linkage. The release of  $\text{Cl}^-$  or  $\text{F}^-$ , inorganic pyrophosphate, and base from the sugar could be accounted for by generation of a 3'-ketone intermediate (Stubbe and Kozarick, 1980a; 1980b). Generation of a 3'-ketone would labilize the 2'-H and 4'-H of ribose, which after elimination would yield a reactive, unsaturated ketone. This reactive intermediate is then proposed to inactivate protein B1. Ketone generation must involve 3'-H loss, presumably via a radical-cation mechanism. Spectroscopic evidence for the generation of a ketone-containing intermediate has been obtained (J. Stubbe, personal communication).

As in the L. leichmannii reductase system, evidence for 3'-H cleavage during the reduction of ribonucleoside diphosphates by E. coli ribonucleotide reductase has been reported (Stubbe and Ackles, 1980). A selection effect against  $^3\text{H}$  of approximately 3.3 has been observed using  $[3'\text{-}^3\text{H}]\text{-UDP}/[^{14}\text{C}]\text{-UDP}$  as enzyme substrate. The decrease in the  $^3\text{H}/^{14}\text{C}$  ratio for reaction product as compared to substrate demonstrates 3'-H cleavage. In addition,  $[3'\text{-}^2\text{H}]\text{-UDP}$  was converted to  $[3'\text{-}^2\text{H}]\text{-dUDP}$  by the E. coli enzyme. These results indicate that cleavage of the 3'C-H bond occurs during the reduction reaction. Thus direct cleavage of the 2'-OH by an  $\text{S}_{\text{N}}1$  or  $\text{S}_{\text{N}}2$  reaction at the 2' carbon is improbable. Similar results were obtained with  $[3'\text{-}^3\text{H}]\text{-ADP}$  and  $[\text{U-}^{14}\text{C}]\text{-ADP}$  (Ator et al., 1982).

Based on this information, a model for the apparently unusual reduction mechanism has been proposed (Stubbe and Ackeles, 1980)(Fig-

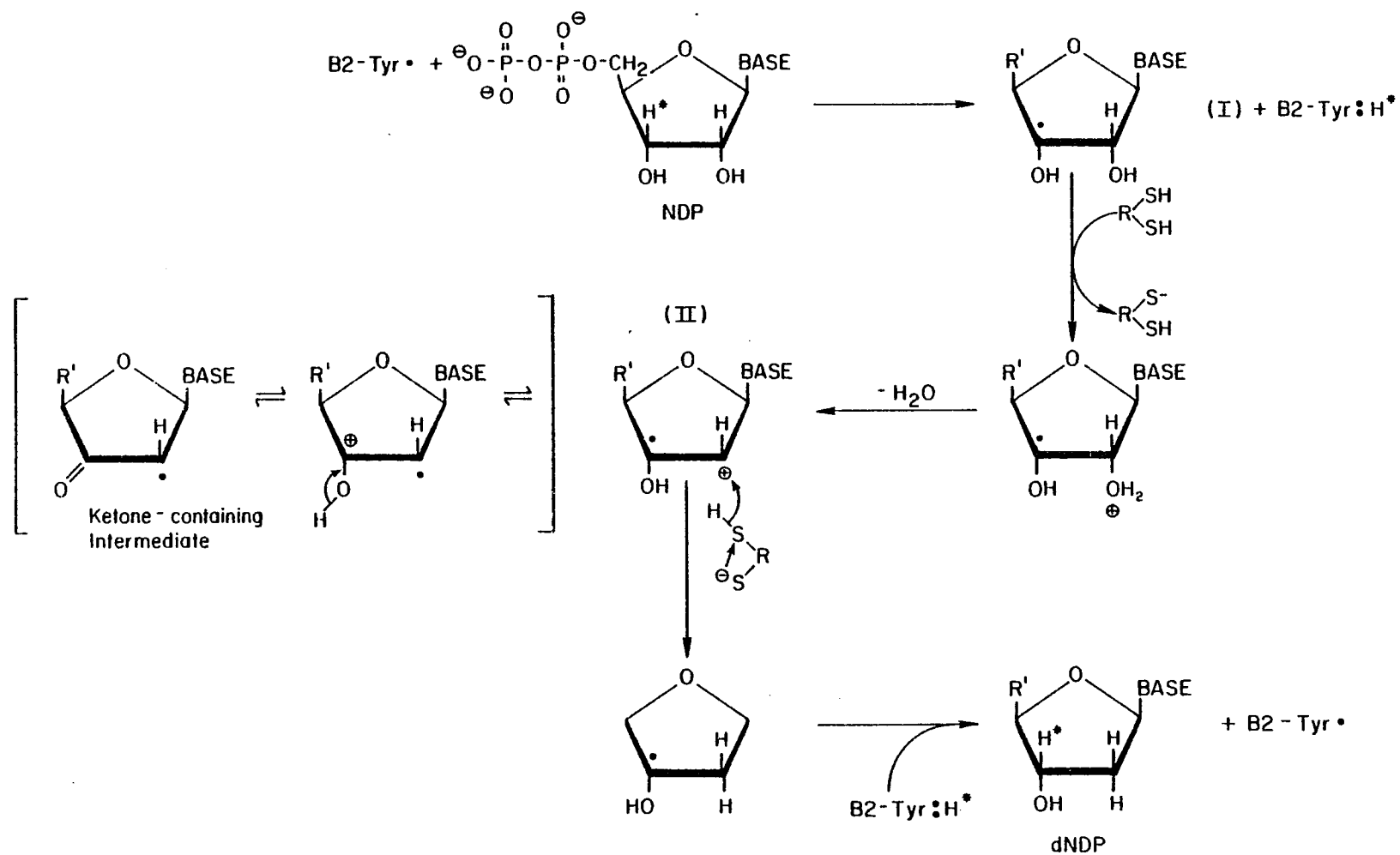
ure 7): The protein B2 tyrosyl radical may abstract a 3'-H from the ribose to generate (I). A more stable radical-cation species (II) would then be formed via the loss of 2'-OH (protonated). Reduction of (II) at the 2' position by thiols on protein B1 would regenerate the 3'-nucleotide radical. The hydrogen atom (originally 3'-H) is re-abstracted from the tyrosyl radical to form product and regenerate the protein B2 radical.

This hypothetical mechanism is also compatible with the  $B_{12}$ -dependent ribonucleotide reductase mechanism previously discussed. Both the E. coli and L. leichmannii enzymes contain a free radical, reactive thiols, and catalyze 3'-H cleavage. In the  $B_{12}$ -dependent system,  $^3\text{H}$  release to the solvent from the  $[3'\text{-}^3\text{H}]$  substrate does not occur (Stubbe et al., 1981), whereas in the E. coli enzyme system a small but significant amount (0.6% at 60% extent of reaction) of  $^3\text{H}$  release is observed (Stubbe and Ackeles, 1980). Thus, the proposed mechanism for ribonucleotide reductase may require modification to account for the minor volatilization of 3'-H from substrate in the E. coli reductase system.

#### Mammalian Ribonucleotide Reductases

Ribonucleotide reductases from mammalian sources have been more difficult to purify. However, the enzymes from rabbit bone marrow (Hopper, 1978), calf thymus (Engström et al., 1979), Ehrlich tumor cells (Cory et al., 1980), and regenerating rat liver (Youdale and MacManus, 1981) have been purified essentially to homogeneity. Some of the properties of the mammalian enzymes are similar to those of the E. coli ribonucleotide reductase. Mammalian reductases are composed

Figure 7. Proposed mechanism for ribonucleoside diphosphate reductase from E. coli (Stubbe and Ackeles, 1980).



of two distinct subunit types which together form active enzyme but are inactive alone. The regulatory (M1) subunit binds both substrate and allosteric effectors while the complementary catalytic (M2) subunit is inactivated by both free radical scavengers, e.g., hydroxyurea and by iron chelators. The pattern of allosteric control for the mammalian enzymes is similar to that observed with the E. coli ribonucleotide reductase (Eriksson et al., 1979; Chang and Cheng, 1979).

The molecular weights of the mammalian enzymes and their constituent subunits differ from the values reported for the E. coli enzyme. In addition, the molecular weights vary with source. Polypeptide molecular weights reported for the M1 and M2 subunits respectively include 84,000 and 110,000 in calf thymus (Engström et al., 1979; Thelander et al., 1980), 127,000 and 75,000 in Ehrlich Tumor cells (Cory et al., 1980), and 100,000 for each in regenerating rat liver (Youdale and MacManus, 1981).

#### Calf Thymus Ribonucleotide Reductase

The most well characterized mammalian ribonucleotide reductase is from calf thymus. A successful purification method for intact enzyme was designed which minimizes the separation of the subunits (Engström et al., 1979). This strategy is in contrast to that used for the bacterial enzyme in which the subunits were purified separately. However, the complementary M2 subunit in intact calf thymus enzyme preparations is obtained in sub-stoichiometric amounts. It is not known if the M2 subunit is preferentially lost during the purification procedure, or alternatively, is present in the cell in nonstoichiometric amounts (Engström et al., 1979).

## Structural Features

### M1 Subunit

The regulatory (M1) subunit of calf thymus ribonucleotide reductase has been purified to homogeneity (Thelander et al., 1980; Plumer, 1980). In the absence of allosteric effectors, protein M1 behaves as a monomer (5.7 s) with a molecular weight of 84,000. Physical characteristics of this subunit are analogous to B1 from E. coli.

Protein M1 contains two binding sites for dATP ( $K_d = 3 \times 10^{-7}$  M) and one binding site for dTTP ( $K_d = 2 \times 10^{-6}$  M) per 170,000 molecular weight (dimer). Both effectors bind to the M1 subunit in a cooperative manner. Equilibrium dialysis experiments also indicate a competition between the binding of nucleotide effectors to these sites. ATP competes with dATP for binding to both sites, but a 2000 fold molar excess of ATP is required to block one of the two dATP binding sites. Furthermore, the presence of dTTP or dGTP decreases the binding of dATP to M1. At 50 fold and 10 fold molar excess for dTTP and dGTP respectively, the binding of dATP to protein M1 was decreased by approximately 50% in both cases. A six fold molar excess of dGTP reduced dTTP binding to protein M1 by 90%, whereas a 500-fold molar excess of ATP only reduced dTTP binding by 70% (Thelander et al., 1980). These experimental observations indicate the presence of two kinds of effector binding sites, one type specific for ATP and dATP, and the other type capable of binding ATP, dATP, dTTP or dGTP. Thus a common property of protein M1 (calf thymus) and B1 (E. coli) is the presence of two different classes of effector binding sites on the regulatory subunit. The bacterial subunit contains two h-type sites



(dATP, ATP, dGTP, dTTP binding) and two l-type sites (ATP or dATP binding) per protein B1 dimer. In contrast, however, protein M1 contains only a total of two binding sites per dimer. Therefore, the observed cooperative binding effects, appear to be significant in the mammalian enzyme system. Conversely, cooperative effects except those involving ATP binding during effector binding have not been observed for the E. coli ribonucleotide reductase (Brown and Reichard, 1969b; Thelander et al., 1980).

The binding of nucleotide effectors to calf thymus protein M1, as with the E. coli system, causes changes in the hydrodynamic properties of the subunit. In solution, the M1 subunit behaves as a monomer (5.7s). Addition of dTTP leads to dimer (8.8s) formation. The presence of dATP induces tetramer (15.2) formation. However, in the presence of ATP, protein M1 exists as a mixture of both dimers and tetramers. Unlike the case with the E. coli B1 subunit,  $Mg^{2+}$  ion did not affect the sedimentation properties of calf thymus protein M1 (Thelander et al., 1980).

### M2 Subunit

The catalytic (M2) subunit has proven to be quite difficult to obtain in homogeneous form. The reported lability of the presumed iron-containing subunit has been a major obstacle during purification (Moore, 1977). The absence of substrate or effector binding sites on the subunit prevent its isolation by affinity chromatography. Two methods have been used to prepare partially purified M2 subunit (Thelander et al., 1980; Plumer, 1980). Both protocols take advantage of allosteric effector binding sites on protein M1. By removing the

M1 subunit from complete enzyme, it is possible to separate the complementary M2 subunit.

Chromatography of partially purified calf thymus enzyme on Blue Sepharose which binds the M1, but not the M2 subunit, was used for the separation of these non-identical proteins (Thelander et al., 1980). Protein M2 activity (5.6s) eluted with a molecular weight average of 110,000 and  $r_s$  of 48 Å during chromatography on Ultragel AcA34. This species is reported to be a dimer of the 55,000 molecular weight protein M2 component, which occurs in sub-stoichiometric amounts in highly purified intact enzyme preparations (Engström et al., 1979). Alternatively, the binding of M1 to DEAE-cellulose can be enhanced in the presence of ATP and  $Mg^{2+}$  ion, allowing for the separate elution of each subunit type from the column when starting with partially purified intact enzyme (Plumer, 1980). Under these conditions protein M2 eluted from the column at a lower ionic strength than protein M1. The activity of crude preparations of protein M2 was associated with a molecular weight species of 87,000 and 100,000 during gel chromatography on Biogel A 1.5 M and Sephacryl S-300, respectively. Each chromatographic system yielded similar results for the Stoke's radius of protein M2, 35.9 Å and 35.8 Å (Plumer, 1980).

#### Comparison of M2 and B2 Subunit Types

The requirement of non-heme iron for activity of the calf thymus ribonucleotide reductase has been described (Engström et al., 1979). Treatment of intact enzyme with EDTA causes inactivation which was reversed by re-addition of iron as  $Fe(NH_4)(SO_4)_2$  or as an Fe(II) ascorbate complex. In contrast to the E. coli enzyme,  $MnCl_2$  reacti-

vated the calf thymus protein almost as well as iron (Brown et al., 1969; Engström et al., 1979). Intact ribonucleotide reductase re-activated with  $^{59}\text{Fe}$  was applied to a glycerol gradient. A good correlation was observed between the peaks of radioactivity and enzyme activity sedimenting through the gradient. However, direct iron analysis of intact enzyme yielded a value of about 0.03 gram atoms of iron per mole of enzyme assuming an enzyme molecular weight range of 200,000-300,000 (Engström et al., 1979). This result was consistent with the observed substoichiometric amount of the 55,000 molecular weight polypeptide (M2), which is obtained in intact enzyme preparations (Engström et al., 1979).

The unique role of protein M2 as the iron-containing subunit of ribonucleotide reductase was further established by treatment of partially purified isolated M2 subunit with EDTA followed by gel filtration to remove chelating agent and any metal-chelate complexes (Plumer, 1980). When protein M2 was assayed in the presence of EDTA-treated protein M1, no significant difference in enzyme activity was observed as compared to the control. However, treatment of protein M2 with EDTA resulted in an apo-M2 protein preparation which when added back to protein M1 had no catalytic activity (Plumer, 1980). The successful reconstitution of enzyme activity with apo-M2 protein by the addition of iron and M1 subunit has not been reported. Iron can be used to activate preparations of intact apoenzyme. Apparently, the presence of protein M1 during treatment with EDTA stabilizes the complementary M2 subunit and facilitates cofactor reconstitution.

Other significant differences between proteins B2 from E. coli and M2 from calf thymus do exist. Hydroxyurea inhibits DNA synthesis in both eukaryotic cells and in E. coli by interfering with ribonucleotide reduction (Castellot et al., 1978). The drug destroys the free radical of protein B2 in E. coli (Atkin et al., 1973). Hydroxyurea also inhibits calf thymus ribonucleotide reductase to a degree similar to that observed with the E. coli enzyme (Brown et al., 1969a; Engström et al., 1979). However, the inhibition of the mammalian ribonucleotide reductase is unlike that of the bacterial enzyme in that it is reversible (Engström et al., 1979). Using partially purified preparations of isolated M2 subunit treated in a similar fashion, Thelander et al., (1980) have shown the target to be the M2 subunit. Mammalian and E. coli ribonucleotide reductases differ in their sensitivities towards substituted thiosemicarbazones. Although they are effective iron chelators, they do not act by removing iron from the enzyme. Fe-thiosemicarbazone complexes are better enzyme inhibitors than the non-complexed forms (Agrawal et al., 1977; Sartorelli et al., 1977). The E. coli enzyme is unaffected at concentrations which inhibit the Novikoff hepatoma enzyme by 50% (Thelander and Reichard, 1979). 2'-deoxy-2'-azidocytidine diphosphate acts as a  $k_{cat}$  inhibitor of E. coli protein B2 by specifically destroying the free radical during reduction (Thelander et al., 1976). With the calf thymus enzyme, the azidonucleotide acts as a competitive inhibitor of normal substrate CDP, with a  $K_I$  of  $2.3 \times 10^{-5}$  M as compared with the  $K_M$  for CDP of  $1.4 \times 10^{-5}$  M (Engström et al., 1979). Removal of the analogue by gel filtration resulted in fully active enzyme.

The reversible inhibition of calf thymus ribonucleotide reductase by both hydroxyurea and 2'-azidocytidine diphosphate is in strong contrast to the irreversible inhibition these compounds exert upon the E. coli enzyme. This indicates structural differences at the catalytic site and possibly different catalytic mechanisms for the E. coli and mammalian enzymes. In addition, it has been suggested that the mammalian enzyme may not contain a permanent free-radical (Engstrom et al., 1979). For the calf thymus enzyme, the existence of a free-radical could not be demonstrated by EPR spectroscopy (Thelander and Reichard, 1979). This result does not preclude the occurrence of a free radical in calf thymus protein M2. The substoichiometric amounts of protein M2 found in intact enzyme preparations (Engström et al., 1979) may result in an enzyme radical level below the limit of radical detection by EPR spectroscopy. A protein M2-related radical signal has recently been reported for a mammalian ribonucleotide reductase. Hydroxyurea-resistant mouse fibroblast 3T6 cells were shown to overproduce the M2 subunit of ribonucleotide reductase. Packed resistant cells gave an EPR signal at 77 K quite similar to that obtained for the tyrosyl free-radical of E. coli B2 (Åkerblom et al., 1981). Isotope substitution experiments have confirmed that the 3T6 cell radical signal was localized at a tyrosine residue (Graslund et al., 1982). Small differences in signal hyperfine character, as compared to E. coli protein B2, were attributed to a slightly changed geometry of the methylene group in relation to the plane of the tyrosyl aromatic ring. The difference in tyrosine free-radical structure suggests a slight alteration in polypeptide conformation around the residue. A pronounced difference in the microwave saturation

characteristics was observed for the enzyme from fibroblast cells when compared to that of E. coli protein B2. This effect was attributed to either differences in the mode of radical interaction with unspecific paramagnetic ions or the binuclear iron center. The antiferromagnetically coupled iron pair, shown to be present in the E. coli enzyme, has been postulated for the mammalian enzyme (Gräslund et al., 1982). But a free-radical signal attributable to a highly purified mammalian ribonucleotide reductase has not been reported. Thus, the physical properties of and the exact catalytic mechanism involving the mammalian ribonucleotide reductase M2 subunit may in fact be quite different from the E. coli counterpart. The presence of a permanent free radical in a mammalian ribonucleotide reductase may require the presence of other components present in a crude cellular extract but absent from highly purified enzyme preparations.

A common feature of both the calf thymus and E. coli ribonucleotide reductases is the capacity of the single enzyme from either source to reduce the four common ribonucleoside diphosphates. Effector requirements are more distinct for the calf thymus enzyme which catalyzes the reduction of the four substrates at almost identical rates (Eriksson et al., 1979). A summary of  $K_m$  and  $V_{max}$  values for the different substrates of purified calf thymus ribonucleotide reductase is shown in Table IIIa. A list of both positive and negative effectors for the reduction of each substrate is also presented (Table IIIb).

### Hydrogen Transport Systems

Two different hydrogen donor systems have also been identified

TABLE IIIa\*

$K_m$  and  $V_{max}$  Values for the Different Substrates of  
Purified Calf Thymus Ribonucleotide Reductase

Substrates	$K_m$ ( $\times 10^{-5}$ M)	$V_{max}$ (nmol $\text{min}^{-1} \text{mg}^{-1}$ )	effector
CDP	3.0-3.2	22.0	ATP ( $5 \times 10^{-3}$ M)
UDP	10	14.2	ATP ( $5 \times 10^{-3}$ M)
ADP	2.8-5.0	19.6	dGTP ( $5 \times 10^{-4}$ M) + ATP ( $2 \times 10^{-3}$ M)
GDP	4.3-5.0	18.5	dTTP ( $5 \times 10^{-4}$ M) + ATP ( $2 \times 10^{-3}$ M)

(\*from Eriksson et al., 1979)

TABLE IIIb\*

Stimulatory and Inhibitory Effects of Different  
Nucleoside Triphosphates on the Activity of  
Calf Thymus Ribonucleotide Reductase

Substrates	Positive Effector <sup>a</sup> or Effector Combination	Inhibition <sup>b</sup>
CDP	ATP ( $1 \times 10^{-3}$ M)	dATP ( $5 \times 10^{-6}$ M)
UDP		dTTP ( $1 \times 10^{-4}$ M) dGTP ( $1 \times 10^{-4}$ M)
GDP	dTTP ( $5 \times 10^{-6}$ M) + ATP ( $1 \times 10^{-3}$ M)	dATP ( $5 \times 10^{-6}$ M) dGTP ( $5 \times 10^{-5}$ M)
ADP	dGTP ( $5 \times 10^{-6}$ M) + ATP ( $1 \times 10^{-3}$ M)	dATP ( $5 \times 10^{-6}$ M) dTTP ( $5 \times 10^{-5}$ M)

Effector concentrations represent (a) the values for  
half-maximal stimulation, and (b) the values which give  
half-maximal inhibition of the stimulated reaction.

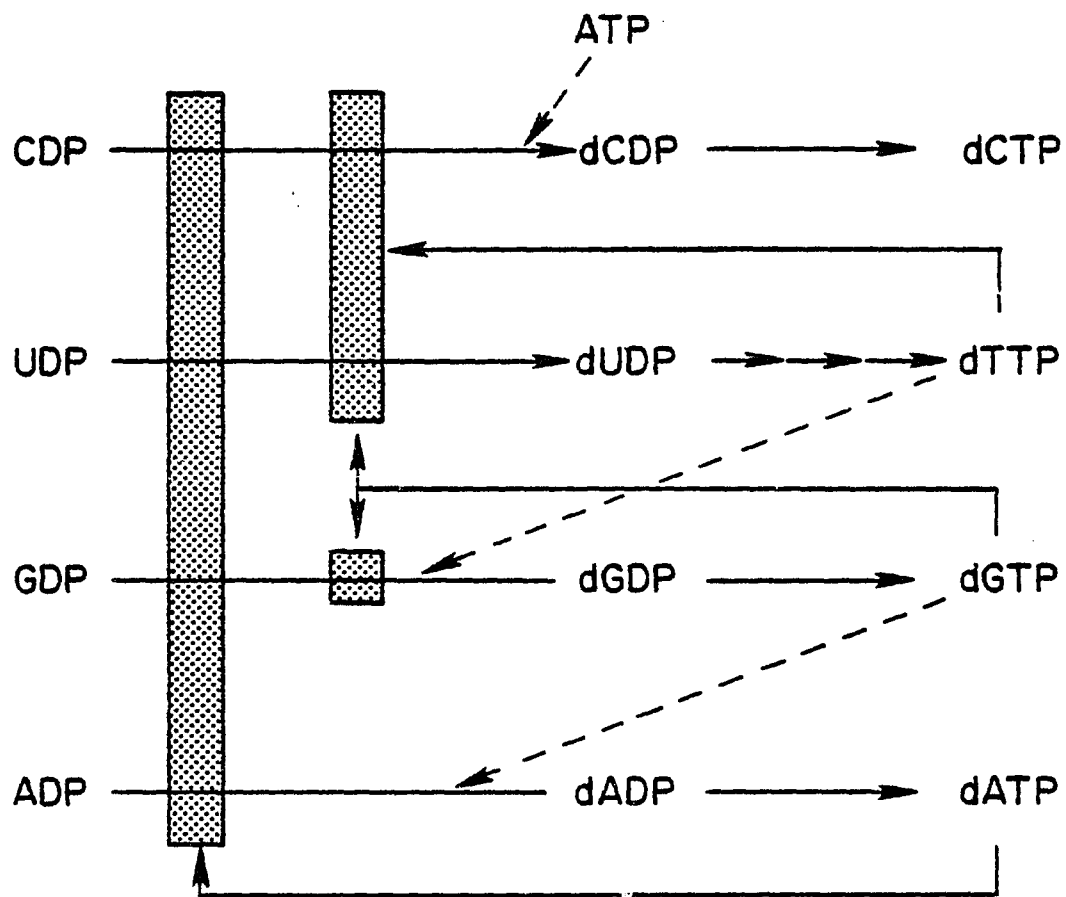
for the calf thymus ribonucleotide reductase system. Thioredoxin from either calf thymus or E. coli does not exhibit species specificity, and is equally active as a hydrogen donor in cross-reactivity experiments (Luthman et al., 1979). Ribonucleotide reductase does show a specificity toward homologous glutaredoxin. Calf thymus glutaredoxin has an apparent  $K_m$  of  $6.0 \times 10^{-7}$  M (at 4 mM reduced glutathione concentrations) with the homologous ribonucleotide reductase, but a value of  $1.9 \times 10^{-6}$  M with the E. coli enzyme (Luthman and Holmgren, 1982). The biological significance of the existence of two potential pathways of hydrogen transport in eukaryotic and prokaryotic tissue remains to be determined.

#### Allosteric Regulation

Kinetic studies have demonstrated that CDP and GDP compete for the same calf thymus ribonucleotide reductase catalytic site (Eriksson et al., 1979). This information, taken together with the ability of the enzyme to catalyze the reduction of all four ribonucleoside diphosphates and the existence of two classes of allosteric effector sites on the M1 subunit (Thelander et al., 1980) has been integrated into a scheme which links in vivo ribonucleotide reduction to DNA synthesis (Figure 8) (Eriksson et al., 1979; Thelander and Reichard, 1979). This general scheme for the effector regulation of ribonucleotide reductase has the following features. The active form of the ribonucleotide reductase always binds ATP. The sequential reduction of CDP and UDP by the ATP-activated enzyme results in the eventual accumulation of dCTP and dTTP in the cell. The increased concentration of dTTP shuts off the reduction of the pyrimidine substrates. The ATP-activated enzyme, in the presence of dTTP, catalyzes the



Figure 8. Scheme for the physiological regulation of ribonucleotide reduction. Broken arrows stand for positive effects, the shaded bars for negative effects. APT is always bound to the active form of the enzyme (Eriksson et al., 1979).



reduction of GDP. Accumulation of dGTP turns off GDP reduction and stimulates ADP reduction. The reduction of ADP is the last step of the proposed sequence. Accumulation of dATP in vivo, e.g. in the absence of DNA synthesis, results in the total suppression of ribonucleotide reductase activity. Since this inhibition is reversible by ATP, the activity of the enzyme in vivo is regulated by the ratio of the adenosine nucleoside triphosphate pools (Eriksson et al., 1979).

Hydroxyurea (Skoog and Nordenskjöld, 1971), thymidine (Bjürsell and Reichard, 1973) and deoxyadenosine (Meuth et al., 1976) have been shown to inhibit DNA synthesis in cultured mammalian cells. The inhibition of DNA synthesis was accompanied by alterations in the intracellular concentration of deoxyribonucleotides. These concentration differences can now be accounted for and are consistent with the allosteric control pattern described above.

Investigations relating to the metabolic pathogenesis of human immunodeficiency disease such as defective T-cell immunity (Giblett et al., 1975) and T-lymphocyte deficiency (Giblett et al., 1972) associated with an inherited deficiency of one or two purine salvage enzymes, have implicated an imbalance in the allosteric control of ribonucleotide reductase as a contributing factor (Cohen et al., 1979). Patients with these disorders are deficient in adenosine deaminase and/or purine nucleoside phosphorylase. The substrates of the missing enzymes, most notably dATP and dGTP, accumulate and have potentially toxic metabolic effects. Ribonucleotide reductase is the proposed target for these toxic deoxyribonucleotides which occur at abnormally

high levels in the patient's lymphocytes (Martin and Gelford, 1981). A response of the ribonucleotide reductase control mechanism, under these conditions would be to cause an imbalance in the deoxyribonucleotide concentrations. This lesion would alter lymphocyte metabolism, function, and proliferation.

Mutants of T-lymphoma cells (S49) in continuous culture have been used as models to study defects in the ribonucleotide reductase control mechanism. A ribonucleotide reductase insensitive to dATP feedback inhibition occurs in a mutant cell line, dGuo-200-1 (Ullman et al., 1980). The intracellular levels of all four deoxyribonucleotides are 2- to 5-fold greater than those found in the parental cell line. The enzyme from the mutant cell line is only half as sensitive to dATP inhibition as is the enzyme from normal cells. The mutant cells were later shown to contain equal amounts of two types of ribonucleotide reductase M1 subunit, one of which was insensitive to dATP inhibition (Eriksson et al., 1981a). Both types of the M1 subunit were purified to near homogeneity by affinity chromatography. Peptide mapping did not demonstrate the nature of the difference between these two regulatory subunit types. However, the abnormal protein M1 is presumably the product of a mutant allele of the protein M1 gene. The existence of two mutant allele products in another S40 cell line (dGuo-L) substantiates the previous conclusion (Ullman et al., 1981). Two altered M1 polypeptides from the dGuo-L cell line were shown to be distinguishable by effector response. While one M1 polypeptide was sensitive to dGTP inhibition and stimulated by dATP, the second M1 polypeptide was insensitive to dGTP inhibition but not inhibited by

dATP. A mutant S49 cell line containing an M1 subunit type resistant to dTTP inhibition has also been described (Eriksson et al., 1982). Competition experiments indicated that the mutation is associated with an allosteric domain defined as the substrate specificity site (h-type) of the M1 subunit. The dGuo-200-1 mutant, which is insensitive to dATP, contains an M1 polypeptide with an altered overall activity (l-type) effector binding site (Eriksson et al., 1981b). Therefore, the occurrence of M1 polypeptide mutants, with altered h- or l-type allosteric site characteristics, provides genetic evidence for independent allosteric domains of protein M1, each responsible for a different aspect of ribonucleotide reductase allosteric control.

#### Ribonucleotide Reductase and DNA Synthesis

In the absence of functional ribonucleotide reductase, the intracellular deoxyribonucleoside triphosphate pools are sufficient only for a few seconds of continuous DNA synthesis (Skoog et al., 1974). The level of enzyme activity is directly related to the rate of cell growth (Thelander and Reichard, 1979). The complex allosteric control pattern of ribonucleotide reductase allows for the balanced production of precursors for DNA synthesis, but does not account for changes in the enzyme activity as a function of cell growth rate. Variations in the activity of ribonucleotide reductase known to occur during the cell cycle (Murphree et al., 1969; Lin et al., 1980) have recently been shown to result from a non-coordinate control of the levels of the complementary M1 and M2 subunits (Eriksson and Martin, 1981; Cory and Fleischer, 1982). In Ehrlich tumor cells, a decrease in CDP reductase activity and cell proliferation in vivo was related

to a decrease in the amount of protein M1 in the cells. In regenerating rat liver, the maximal level of reductase activity occurred 36 hours after partial hepatectomy while the level of protein M2 activity was maximum at 24 hours. Therefore, the components of ribonucleotide reductase were not coordinately varied until the point of maximal enzymatic activity (Cory and Fleischer, 1982).

Additional evidence of a non-coordinate control of ribonucleotide reductase subunit activity has been obtained using cultured mouse lymphoma (S49) cells. Treatment of cells with dibutryl-cAMP arrests S49 cells in the G1 growth phase (Coffino et al., 1975). Cells arrested in G1 showed only 10 to 20% of the normal ribonucleotide reductase activity. Addition of exogenous M2 subunit, but not M1, caused a four-fold stimulation of enzyme activity in the extracts from G1-arrested cells. Thus, the activity of protein M2 was decreased in G1-arrested cells in parallel with the decrease of intact enzyme activity (Eriksson and Martin, 1981). A population of exponentially growing S49 cells was also separated into a 90% pure G1 cell population, a mixture of G1 and early S phase cells, and a 95% pure S/G2 phase cell population by centrifugal elutriation. The distribution of DNA content per cell was analyzed by flow cytometry and the amounts of complementary ribonucleotide reductase subunits determined for each cell population. The specific activity of protein M1 remained the same in each cell population, but that of M2 was decreased by 60% in the G1 population (Eriksson and Martin, 1981). This suggests that the non-coordinate control of ribonucleotide reductase activity involves a cell cycle-dependent variation in the activity of the M2 subunit.

These results may also explain the apparent sub-stoichiometric amounts of protein M2 obtained in preparations of intact ribonucleotide reductase from calf thymus (Engstrom et al., 1979). Thymus tissue may contain a large proportion of cells in the resting (G1) phase of the cell cycle (Eriksson and Martin, 1981).

The level of mammalian ribonucleotide reductase activity in vivo appears to be modulated by both a complex allosteric control pattern and a non-coordinate cell cycle-dependent variation in complementary subunit levels. The metabolic control exerted on and by ribonucleotide reductase may be of even greater significance if the enzyme is part of a multiprotein complex for DNA replication. Ribonucleotide reductase from E. coli (Lunn and Pigiet, 1979), T4 infected E. coli (Mathews et al., 1979; Allen et al., 1980), and Chinese hamster embryo fibroblast cells (Reddy and Pardee, 1981) has been found associated with other enzymes of DNA replication in a high molecular weight complex. This complex or "replicase" would be capable of efficient and economical channeling of deoxyribonucleotides to the DNA replication fork in a manner not feasible for independent soluble enzymes. Also, control of the DNA synthetic rate by allosteric modulation of "replicase" enzymes would be more direct and efficient.

### Perspectives

The ribonucleotide reduction step is a logical target point in the design of various cancer chemotherapeutic agents, which function as enzyme inhibitors. Ribonucleotide reductase inhibition results in a rapid and almost total inhibition of DNA synthesis (Skoog and

Nordenskjöld, 1971; Cory and Mansell, 1976) and a decrease in the rate of cell growth (Thiess and Fischer, 1976). Several compounds have been evaluated as potential drugs in clinical trials, including hydroxyurea (Thurman, 1964), and the  $\alpha$ -(N)-heterocyclic carboxaldehyde thiosemicarbazones (Sartorelli et al., 1968; French et al., 1974). Hydroxyurea unfortunately has a low potential for in vivo use because it is a weak in vitro ribonucleotide reductase inhibitor (Elford, 1968). This drawback necessitates the administration of frequent and high doses for effective antineoplastic activity. The thiosemicarbazones, which are potent ribonucleotide reductase inhibitors, are ineffective and toxic in humans (deConte et al., 1972; Krakoff, 1974). Chemotherapeutic agents, directed against ribonucleotide reductase, currently being studied include imidazopyrazole (IMPY) compounds (Brockman et al., 1978), polyhydroxybenzene derivatives (Elford et al., 1981), and combinations of selected antineoplastic agents (Cory et al., 1981).

A biophysical understanding of the enzyme's structure, function, and regulation is prerequisite to the design of specific inhibitors. Drugs such as these, unlike the chemotherapeutic agents directed at alternative metabolic steps, might effectively attenuate uncontrolled cell proliferation by influencing only one metabolic conversion. Compounds which affect both complementary subunits are currently being evaluated in other laboratories. However, a lack of physical information regarding the M2 subunit has complicated inhibitor selection and design.



The B2 subunit of the E. coli ribonucleotide reductase exhibits a UV-visible absorption spectrum which is remarkably similar to that observed for oxyhemerythrin. Spectral similarities between E. coli protein B2 and hemerythrin are considered to be related to conservation of iron binding domain characteristics. The apparent iron center homology between two proteins with very different biophysical and functional character is of great interest, and implies functional domain conservation. The possibility exists that protein M2 may also contain an analogous dimeric iron center. To gain insight on these and other problems pertaining to the biophysical nature of the mammalian ribonucleotide reductases, more M1 structural information, and a purification method for and a characterization of M2 are required.

## II. MATERIALS

Thymus tissue from 2-6 month old calves was obtained from a local slaughterhouse (J.T. Trelegan Co., Cambridge, MA). The fresh tissue was frozen and stored at  $-85^{\circ}\text{C}$ . dATP-Sepharose was synthesized by Debra Ward (Ward, 1980) according to Berglund and Eckstein (1974) as modified by Knorre et al. (1976). Highly purified E. coli ribonucleotide reductase was the generous gift of Dr. JoAnne Stubbe (University of Wisconsin, Madison).

All chemicals were reagent grade or the best grade commercially available. All buffers were prepared with water purified by reverse osmosis, deionization, and filtration sterilization (Milli-Q, Millipore Corporation, Bedford, MA).

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### III. METHODS

#### Ribonucleoside Diphosphate Reductase Assay

The assay for ribonucleoside diphosphate reductase activity was based on a modification of procedures described by Cory and Mansell (1975), Larson (1969), and Moore (1967). The concentrations of ATP and  $\text{Mg}(\text{CH}_3\text{COO}^-)_2$  used in the assay were optimized for the reduction of the cytidine diphosphate (CDP) substrate (Plumer, 1980). The substrate, 5- $^3\text{H}$ -cytidine-5'-diphosphate ( $^3\text{H}$ -CDP) (New England Nuclear), was stored at  $-85^\circ\text{C}$  prior to use. Unlabeled CDP was added to give a final specific activity of 33 uCi/umol.

CDP reductase activity was assayed in a final volume of 120 ul containing 7.7 mM potassium phosphate buffer, pH 7.0 which was 0.1 mM in CDP, 6.2 mM in dithioerythritol (DTE), 3.3 mM in ATP, 2.0 mM in  $\text{Mg}(\text{CH}_3\text{COO}^-)_2$ , 8.3 mM in NaF, 0.06 mM in  $\text{FeCl}_3$ , and contained 0.4 uCi of  $^3\text{H}$ -CDP (33 uCi/umol). Assay mixtures were incubated at  $37^\circ\text{C}$  for twenty minutes and the reaction terminated by placing the tightly capped assay tube in a boiling water bath for 5 minutes followed by a brief centrifugation. Control values were obtained by incubating 100 ul of a boiled enzyme preparation with the standard assay components. All assays and controls were performed in duplicate.

The product of CDP reductase activity, 2'-deoxycytidine-5'-diphosphate (dCDP) was hydrolyzed to the deoxyribonucleoside by snake venom phosphodiesterase I (Crotalus atrox venom). To each assay tube, in a total volume of 100 ul, the following components were added: 1 mg of snake venom (Sigma), 0.288 nmole of 2'-deoxycytidine-5'-mono-

phosphate, and 1.0 nMole of  $\text{MgCl}_2$  in 0.03 M Tris-HCl buffer, pH 9.0. After addition of the hydrolysis mixture, each assay tube was incubated at  $37^\circ\text{C}$  for 2.5 hours. After incubation, the reaction was again terminated as described above, and brought to a final volume of 1.0 ml by addition of water. The assay tube was then centrifuged to sediment denatured protein and the supernatant applied to a column of Dowex-1-borate, to separate the deoxy- and ribonucleosides (Steeper and Stuart, 1970). 2'-deoxycytidine was completely eluted from the Dowex-1-borate column with 4.0 ml of water. A 1.0 ml aliquot of the effluent was placed in a scintillation vial with 10 ml of Scint-A<sup>TM</sup> (Packard Instrument Co., Dovers Grove, IL) scintillation cocktail. Radioactivity was determined by counting in a Beckman LS7000 scintillation counter.

One unit of ribonucleotide reductase activity is defined as that amount of enzyme which converts 1.0 nmole of CDP to dCDP per minute at  $37^\circ\text{C}$  under the conditions described.

#### Protein Determination

Protein was determined by using Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories) as described by Bradford (1976).

#### Purification of Intact Ribonucleotide Reductase

The procedure for the preparation of highly purified intact ribonucleotide reductase was patterned after that of Engström et al., (1979) but was simplified by eluting the DEAE-cellulose column in a stepwise manner (Plumer, 1980), deleting the hydroxylapatite column, and replacing the gel chromatography desalting step with dialysis.

All purification procedures were carried out at about 4°C, except for the dATP-Sepharose step which was performed at 25°C. A protein determination and a ribonucleotide reductase assay were performed at each step of the procedure. A schematic outline of the steps involved is shown in Figure 9.

#### Homogenization

Approximately 1.5 kg of frozen (-85°C) calf thymuses were broken into small pieces, roughly one cubic inch and placed in 2.6 l of 50 mM Tris-HCl, 0.1 mM DTE pH 7.6 (Buffer A) at room temperature. The tissue was allowed to partially thaw, with occasional stirring, at room temperature for one hour. After thawing, tissue homogenization was carried out at 4°C in a stainless steel Waring blender. The homogenate was centrifuged at 13,000 X g for 40 minutes to remove insoluble material. The supernatant was filtered through glass wool to remove lipid material.

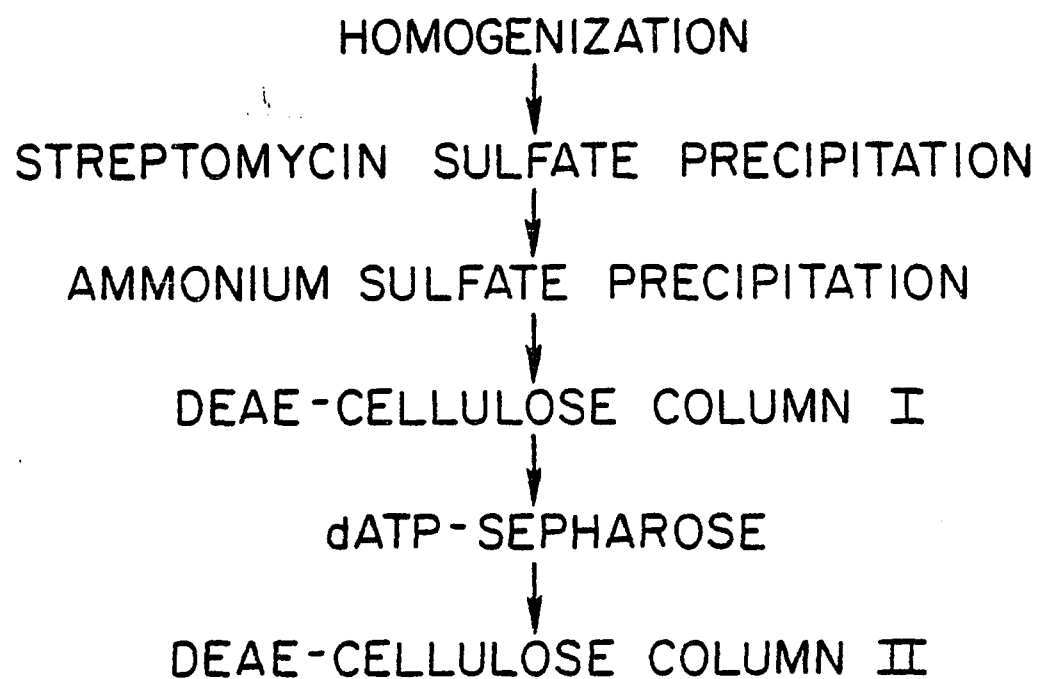
#### Precipitation with Streptomycin Sulfate

2.0% (w/v) streptomycin sulfate in buffer A was slowly added to the filtrate to give a final concentration of 0.5%. The mixture was stirred on ice for 20 minutes. After standing an additional 10 minutes, the suspension was centrifuged as described above.

#### Precipitation with Ammonium Sulfate

Solid ammonium sulfate (Schwartz-Mann, ultrapure) was added to the streptomycin sulfate supernatant to a final concentration of 40%. Upon dissolution, the sample was allowed to stand an additional 10 minutes before centrifugation at 13,000 X g for 40 minutes. The pellets were dissolved in Buffer A (15-20% of original volume). Equal

Figure 9. Scheme for the purification of the complementary subunits of ribonucleotide diphosphate reductase from calf thymus.





aliquots were placed into three dialysis bags (Spectrapor 2, Spectrum Medical Industries, Inc., Los Angeles). Each bag was then dialysed overnight against 4 liters of Buffer A with one buffer change. Insoluble material was removed from the dialysate by centrifugation at 19,500 X g for 10 minutes.

#### DEAE - Cellulose Chromatography

The supernatant of the centrifugated dialysate was applied to a 5 X 45 cm DEAE-cellulose column (DE-52, Whatman) which had been previously equilibrated with Buffer A. The flow rate was maintained at 4 ml/min until the sample was loaded. The sample was followed by 1 l of Buffer A and the flow rate increased to 5 ml/min. The eluent was then changed to 2 l of Buffer A, 0.05 M KCl. Ribonucleoside diphosphate reductase activity was then eluted with 3 l of Buffer A, 0.1 M KCl (Buffer B). The first 840 ml of this eluent ( $\sim V_0$ ) was discarded and the remaining fractions pooled into 4 equal aliquots designated fractions A, B, C, and D. Each pool (volume  $\sim 500$  ml) was made 80% saturated in ammonium sulfate and centrifuged at 13,000 x g for 40 minutes. The pellets were resuspended in a minimal volume of Buffer A (5-15 ml) and dialyzed against 4 liters of Buffer A overnight with one buffer change. Following dialysis the samples were quickly frozen in dry ice/acetone and stored at  $-85^{\circ}\text{C}$ .

#### dATP-Sepharose Chromatography

Those pools from the previous step containing the highest ribonucleoside diphosphate reductase activity (1.5-4.0 units/ml) were thawed, combined, and made 0.1 M in KCl. A minimum of 55 units were centrifuged at 3,000 x g for 10 minutes. Small aliquots of the super-

natant (~ 2.5 ml) were individually warmed to 25°C by swirling in a 37°C water bath and degassed. Successive aliquots were applied to a 0.9 X 2.0 cm column of dATP-Sepharose which had been equilibrated with Buffer B at 25°C. The flow rate was 6.0 ml/hour. Following sample application, the column was eluted with Buffer B until the absorbance of the effluent at 280 nm was less than 0.05. The column was then eluted with Buffer B containing 0.5 mM ATP until the effluent absorbance at 295 nm was less than 0.05. Intact ribonucleotide reductase was then eluted with 7.5 ml of Buffer B containing 50 mM ATP. All fractions were collected on ice. The Buffer B containing 50 mM ATP eluent (7.5 ml) was brought to 80% saturation in ammonium sulfate by addition of solid and centrifuged at 13,200 X g for 40 min. The precipitate was resuspended in a minimal volume (1 ml) of Buffer A. The intact ribonucleotide reductase sample was then dialyzed against 4 l of Buffer A overnight, frozen in dry ice/acetone, and stored at -85°C or separated into its component subunits in the next step.

The dATP-Sepharose (1.5 ml) was regenerated after each use by washing the resin with 20 ml of 6 M guanidinium-HCl (sequanal grade, Pierce) followed by a prolonged wash with 50 mM Tris-HCl, pH 7.6 as previously described (Ward, 1980).

#### Chromatography on DEAE Cellulose in the Presence of ATP and $\text{Mg}(\text{CH}_3\text{COO})_2$

Separation of the component subunits, M1 and M2, of purified intact ribonucleotide reductase was accomplished by an adaptation of a procedure which had previously been used to separate the activity of the complementary subunits in crude preparations (Plumer, 1980).

A 0.9 X 2.3 cm DEAE-cellulose column (DE-52, Whatman) was equilibrated at 0-4°C with 0.05 M Tris-HCl buffer, pH 7.6, which was 0.10 mM in DTE, 4 mM in ATP, and 4 mM in  $\text{Mg}(\text{CH}_3\text{COO}^-)_2$  (Buffer C). Purified intact ribonucleotide reductase (25-60 units) in Buffer C was made 0.05 M in KCl and allowed to stand at 0°C for 1 hour. The sample was applied to the column at 6 ml/hr. It was then eluted with about 10 ml of Buffer C containing 0.1 M KCl. When the absorbance of the fractions (1.5 ml) at 295 nm had returned to zero, 10 ml of Buffer C containing 0.25 M KCl was applied to the column. Subunit M2 eluted in the 0.1 M KCl containing buffer, while subunit M1 is found in the 0.25 M KCl fractions. The purified subunits were concentrated by precipitation with 80% saturated ammonium sulfate followed by centrifugation at 13,200 X g, 40 minutes. The pellets were then dissolved in 0.5-1.0 ml of Buffer B and dialysed against 4 l of the same buffer for 4 hours with one change after two hours. Aliquots were removed for assay and protein determination, and the M1 and M2 subunit fractions were frozen in dry ice/acetone and stored at -85°C.

#### SDS-Polyacrylamide Gel Electrophoresis

Preparations of intact ribonucleotide reductase and its purified subunits were electrophoresed in tube gels using the method of Weber and Osborn (1969) or in 11-14% exponential gradient slab gels using the discontinuous buffer system of Laemmli (1970) as described by O'Farrell (1975). Samples for slab gel application were made 10% (w/v) in glycerol, 5% (v/v) in  $\beta$ -mercaptoethanol, 2.3% in SDS and 62.5 mM in Tris-HCl pH 6.8 (SDS sample buffer) and denatured at 100°C for 10 minutes. Samples containing less than 600 ug protein/ml were prepared for electrophoresis by addition of an excess volume of SDS

sample buffer (15-20 fold). The samples were then denatured, and concentrated in a disposable multiwell Minicon-CS15 concentrator (Amicon, Danvers, MA) to a volume appropriate for gel application. Molecular weight standards were run simultaneously for  $M_r$  comparisons. The SDS gel calibration standards (Pharmacia) contained the following proteins: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and  $\alpha$ -lactalbumin (14,400). Typically 5 ug of each sample and 5 ug of each standard component was applied to each gel lane.

In preparing exponential gradient gels, a volume of 10.7 ml was used in the front chamber of the gradient mixer. The separating gel volume was 29.5 ml (O'Farrell, 1975). The overall separating gel dimensions were 12.5 X 14 X 0.15 cm. Electrophoresis was carried out at 30 mA constant current/slab for approximately 3.5 hours. Gels were stained for a minimum of 4 hours with 0.1% ( $W/v$ ) Coomassie Brilliant Blue R-250, 9.1% ( $V/v$ ) acetic acid and 45.5% ( $V/v$ ) methanol, and diffusion destained in 5% ( $V/v$ ) methanol, 10% ( $V/v$ ) acetic acid. Gels were preserved by drying on a slab gel dryer (Hoeffer, San Francisco, CA).

#### Amino Acid Composition Analysis

The amino acid composition of purified ribonucleotide reductase subunits was determined using a Beckman model 118CL amino acid analyzer interfaced to a Varian model CDS-111C integrator. Individual subunit samples were dialyzed overnight against Milli-Q system water at 4°C to remove buffer salts. Aliquots of each sample were placed in hydrolysis tubes and vacuum dessicated overnight to remove solvent.

Aliquots of dialysate were simultaneously run as a control for possible background contamination.

Samples were hydrolyzed in vacuo in 1.0 ml of constant boiling HCL, 5.7 M (sequanal grade, Pierce) for 24 hours at 110°C. Prior to hydrolysis, a drop of 0.5 M hydrazine was added to prevent tyrosine degradation (Sanger and Thompson, 1963). After hydrolysis, hydrolysis tubes each containing a sample were placed in a vacuum dessicator containing one 400 ml beaker with NaOH pellets and another of concentrated H<sub>2</sub>SO<sub>4</sub> to remove solvent. The dry samples were next dissolved in a minimal volume (<1.0 ml) of 0.2 N sodium citrate pH 2.2 containing 0.5% thiodiglycol and 0.1% phenol (sample dilution buffer) (Beckman Instruments, Palo Alto, CA). Aliquots of the re-dissolved samples were injected onto the amino acid analyzer and chromatographed by single column methodology as described (Spinco Application Note AN-001, 4/77, Beckman Instruments, Spinco Division) using a modification of procedures originally detailed by Hamilton (1963) and Spackman, Stein, and Moore (1958).

Tryptophan was determined with the amino acid analyzer using the hydrolysis procedure of Simpson et al. (1976). Protein hydrolysis was carried out in vacuo in the presence of 4N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Pierce Chemical Co.) for 24 hours at 110°C. After hydrolysis, the contents of the tube were partially neutralized by addition of an equal aliquot of 3.5 N NaOH, and injected onto the analyzer.

The determination of half-cystine as cysteic acid was accomplished by oxidizing the sample protein with performic acid according to Moore (1963) prior to hydrolysis as previously described with omission of the hydrazine.

Corrections for the degradation of threonine and serine were performed by hydrolyzing identical samples for 24, 48, and 72 hours respectively, prior to amino acid analysis. The logarithm of the concentration of these residues was plotted versus hydrolysis time and extrapolated to zero hydrolysis time to correct for losses. Isoleucine and leucine were quantitated by plotting the concentration of each versus inverse time, and extrapolating the line to infinite hydrolysis time.

The analyzer/integrator system was calibrated by chromatographing an amino acid standard reference mixture (Beckman Instruments). For the cysteic acid determination, an aliquot of calibration standard was treated with performic acid and subjected to hydrolysis as described. Recovery of cysteic acid was corrected for losses during manipulation, by normalization to stable components such as leucine or isoleucine.

For the tryptophan determination, an external tryptophan standard was prepared by making a  $6.5 \times 10^{-3}$  M stock tryptophan (Sigma) solution. A 1:50 dilution was made and the ultraviolet absorption spectrum in the region of 250-300 nm measured with a Hitachi Perkin-Elmer Coleman 124 double beam spectrophotometer. The absorbance values at 278 and 272 nm were then employed to determine the actual

concentration of tryptophan in the stock solution. The molar extinction coefficients used were 5579 and 5344 1/M cm (Sober, 1970), respectively. The concentration value indicated by the absorbance measurement was in agreement with the value obtained at the second wavelength, typically by better than 99%. Finally, an appropriate aliquot was added to the sample dilution buffer and the calibration standard to yield a final component concentration reflecting the sensitivity setting of the analyzer (usually 10 nmoles of each amino acid/injection). Also, the pH of the single column methodology Buffer C (1.0 N Na citrate, pH 6.4) and the final column incubation temperature (62-65°C) were altered as required to facilitate the resolution of ammonia and tryptophan, which under less than optimal conditions would not be well resolved.

#### Analytical Peptide Peptide Mapping

Peptide mapping was performed by limited sample proteolysis in SDS (Cleveland et al., 1977) followed by analytical gel electrophoresis. To insure subunit purity, samples (8-15 ug) were first run on an 11-14% exponential gradient SDS slab gel as described. Visualization of the protein bands was performed by placing the gel, immediately after completion of electrophoresis, into 10-20 volumes of 4 M sodium acetate at 25°C. The gels were then agitated by slow reciprocal shaking for 30-60 minutes. Those regions containing protein appeared as transparent bands against a white background of precipitated unbound SDS (Higgins and Dahmus, 1979). Bands of interest were cut from the gel, placed in 1.0 ml of 0.125 M Tris-HCl, pH 6.8 containing 0.1% SDS and 1 mM EDTA, and stored at -20°C until use.

### Limited Proteolysis in SDS

Individual gel pieces were thawed and placed in 10 ml of 0.125 M Tris-HCl, pH 6.8 containing 0.1% SDS and 1 mM EDTA. The slices were equilibrated for 0.5 hours at 25°C. Each slice was then trimmed and placed in a sample well of a second gel, the wells of which had been previously filled with the same buffer. 10 ul of this buffer containing 20% glycerol was used to fill the spaces around the slice. Finally, the sample was overlayed with 10 ul of this buffer containing 10% glycerol and 0.1 ug S. aureus V8 protease (Miles, 36-900-1) (Cleveland et al., 1977). The height of the stacking gel was 5 cm. The separating gel was a 12-18% exponential gradient SDS slab gel poured as described above. Separating gel dimensions were 8.5 X 14 X 0.15 cm. Electrophoresis was performed as before, except that when the tracking dye had reached the bottom of the stacking gel, the current was turned off for 0.5 hours, then re-applied until the tracking dye had migrated to the bottom of the separating gel. Detection of peptides and/or protein in the polyacrylamide gel was performed by staining with Coomassie Blue as previously described.

### M2 Subunit Iron Determination

The iron content of purified ribonucleotide reductase catalytic (M2) subunit was evaluated by the method of atomic absorption spectroscopy. An Instrumentation Laboratories model 951 AA/AE spectrophotometer equipped with a model 655 furnace atomizer system was used throughout this study.

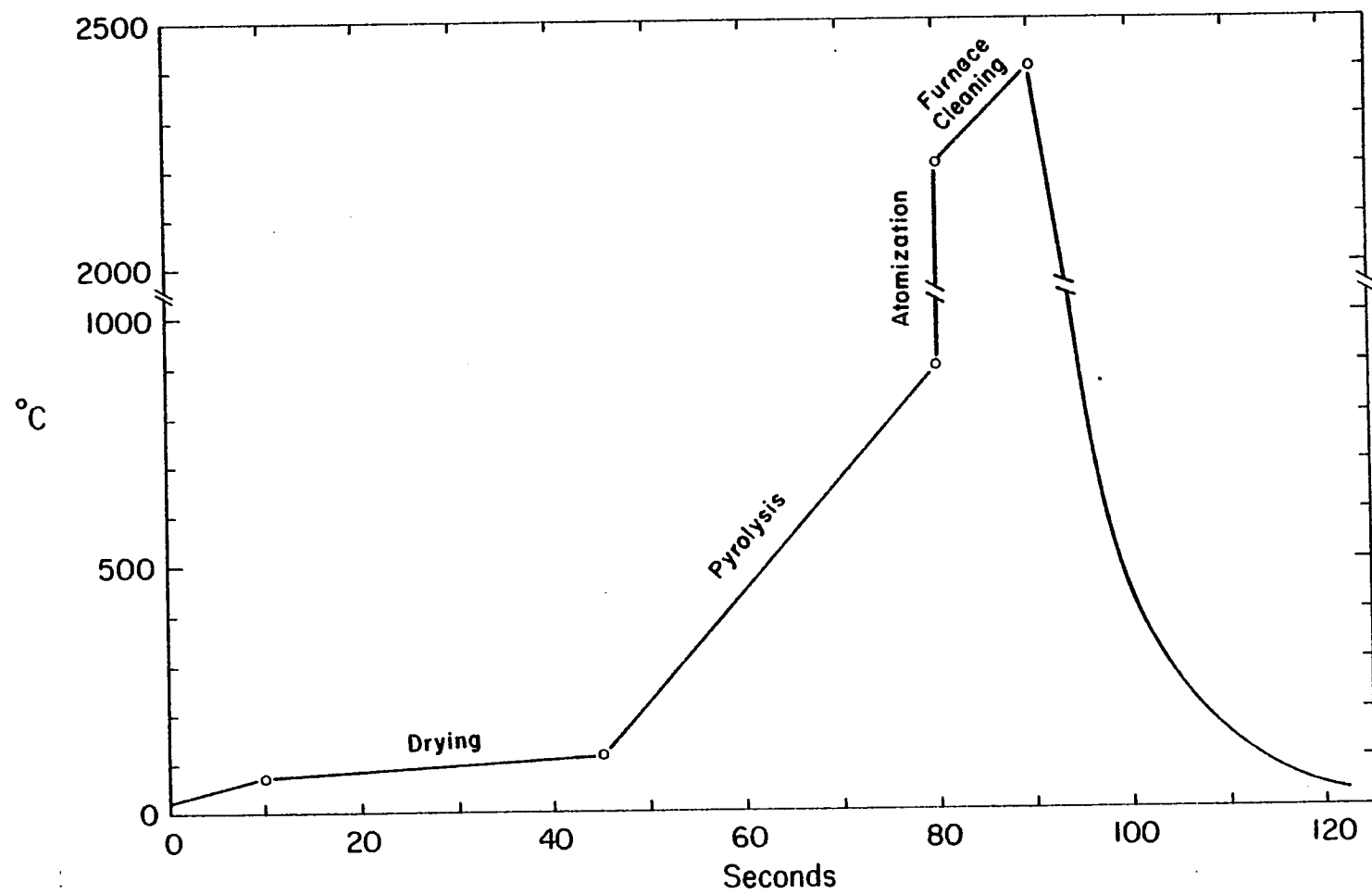
All plasticware used for sample or reagent preparation was carefully cleaned, soaked in 0.5% HNO<sub>3</sub> overnight, copiously rinsed with



Milli-Q system water, and dried prior to use. Samples of catalytic subunit were evaluated as prepared in 0.1 M KCl, 0.1 mM DTE, 50 mM Tris-HCl pH 7.6. In addition, to eliminate a potential background problem of iron coordinated to Tris, aliquots of each sample were buffer exchanged into 0.1 M KCl, 50 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid pH 7.6 (Chelex-100 treated HEPES buffer). Prior to use, 500 ml of 0.1 M KCl, 50 mM HEPES pH 7.6 was passed over a 1.5 X 20 cm column of Chelex-100 (Bio-Rad Laboratories) to remove any contaminating metals. 300  $\mu$ l aliquots of sample were then run on a 0.9 X 3.2 cm column of Bio-gel P-6 (Bio-Rad Laboratories) equilibrated with the Chelex-100 treated HEPES buffer. The entire column assembly was comprised exclusively of polypropylene or teflon materials. The flow rate was 4.0 ml/hr. The effluent was collected in polypropylene test tubes (12 X 75 mm). Prior to sample buffer exchange, the resolving efficiency and elution characteristics of the column system had been first evaluated in an identical situation using a mixture of BSA and  $\text{NaN}_3$  as  $V_0$  and  $V_i$  markers, respectively.

The atomic absorption spectrophotometer was operated in the single beam mode with deuterium background correction. The detector was adjusted to the primary absorbance line of Fe at 248.3 nm. The detector sensitivity was 1 AUFS and the band pass 0.3 nm. A non-coated pyrolytic graphite cuvette (Instrumentation Laboratories) was used in the electric furnace assembly. Cuvette conditioning and cleaning was carried out by repeated (10-15X) furnace program cycling up to 2900°C. The furnace program utilized during sample application, drying, pyrolysis, and atomization is shown in Figure 10.

Figure 10. Furnace program employed during M2 subunit iron content determination using atomic absorption spectrophotometry.

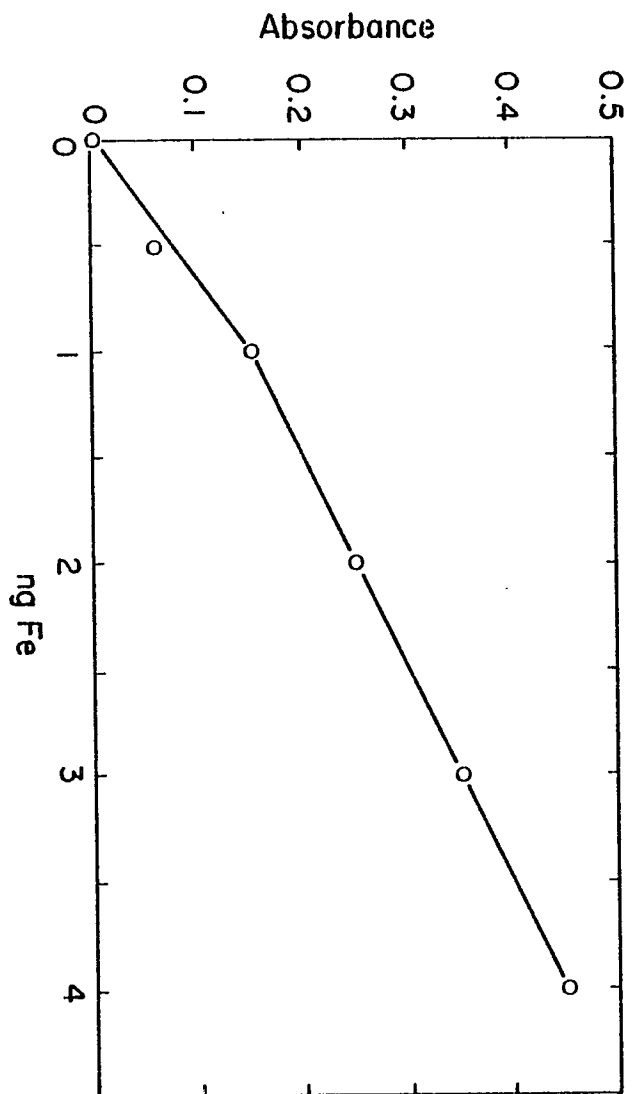


Instrumentation was standardized with a certified atomic absorption Fe reference standard (Fisher). The 1,000 PPM reference solution was diluted with 0.5%  $\text{HNO}_3$  (Ultrex, VWR) to prepare a calibration curve in the operating range utilized (Figure 11). This data was compiled by the spectrophotometer microprocessor, and the instrument then programmed for operation in the auto-calibrate/flex curve mode. The resulting 2 or 3 ng Fe absorbance value was subsequently used as a reference point by the flex curve software to compensate for instrument drift during operation. Standards were run repeatedly during the analysis to correlate the calibration curve slope with changes in instrument sensitivity. Control blanks were repeatedly run to monitor and compensate for any baseline drift using the auto-zero routine.

20 ul volumes of standards and samples were injected into the furnace. Each aliquot was delivered manually from a new RC 20 pipette tip (Rainin certified, Rainin) attached to a Gilson P20 pipetman.

Protein containing samples in either buffer system described were diluted prior to analysis with an equal volume of 1%  $\text{HNO}_3$  (Ultrex, VWR) containing 0.1 M  $\text{NH}_4\text{NO}_3$ .  $\text{NH}_4\text{NO}_3$  was added to facilitate the removal of buffer system KCl, as  $\text{NH}_4\text{Cl}$  and  $\text{KNO}_3$  since these compounds decompose at pyrolytic and not atomization furnace temperatures, and thus remove salt which otherwise might interfere in the atomization, e.g., Fe determination step (Price, 1979). Subsequent sample dilutions were made with 0.5%  $\text{HNO}_3$  (Ultrex) to obtain absorbance values in the linear range of the calibration curve.

Figure 11. Calibration curve for iron standards run on atomic absorption spectrometer. Each point represents an average of four consecutive absorbance values obtained at each standard concentration.



### Gel Filtration Chromatography

Analytical gel exclusion chromatography was performed using a Beckman model 332 high performance liquid chromatograph (HPLC) equipped with an Altex Spherogel TSK-3000SW column (7.5 X 600 mm + 7.5 X 100 mm guard column). The system was equilibrated with 0.25 M KCl, 50 mM Tris-HCl pH 7.6. Chromatography was performed at 0°C using a flow rate of 30 ml/hr. The effluent was monitored at 280 nm with a Hitachi model 100-40 spectrophotometer equipped with an Altex model 155-00 standard analytical flow cell.

Column calibration was performed by chromatographing various native protein standards, alone and as mixtures, under the conditions described above. Standards used to calibrate the column included ferritin (horse spleen, 440,000), gamma-globulin (bovine, 167,000), aldolase (rabbit muscle, 158,000), hexokinase (yeast, 102,000), ovalbumin (hen, 43,000), and trypsin inhibitor (soybean, 20,100). Blue Dextra 2000 (Pharmacia,  $>10^6$ ) and  $\text{NaN}_3$  (65) were used to determine  $V_o$  and  $V_i$ , respectively.

Ribonucleotide reductase subunit samples were thawed and clarified by brief centrifugation in an Eppendorf model 5413 centrifuge (6500 X g, 3 minutes) prior to injection onto the HPLC.

### Determination of Subunit Oligomeric Structure

The oligomeric structure of the purified ribonucleotide reductase catalytic (M2) subunit was determined by chemical cross-linking.

Cross-linking reactions were performed in the presence of 0.1 M KCl to facilitate subunit stability (Thelander et al., 1980). The protein concentration selected was 160 ug/ml to favor crosslinking within protomer over that between protomer (Davies and Stark, 1970).

#### Chemical Cross-Linking with Dimethyl Suberimide

The conditions employed for subunit M2 cross-linking with dimethyl suberimide were according to Hillel and Wu (1977). A 300 ul aliquot of subunit M2 (1mg/ml) in Buffer B was applied to a 0.9 X 3.2 cm column of Bio-gel P-6 which had previously been equilibrated with 0.1 M KCl, 0.1 mM DTE, 50 mM Bicine-KOH pH 8.5 (Reaction Buffer B) and calibrated as described. The flow rate was 4.0 ml/hr, and the column operating temperature was 4°C. The  $\sim V_0$  fractions containing the sample were pooled and the protein concentration determined. The sample was next diluted with Reaction Buffer B to a protein concentration of 175 ug/ml. Immediately prior to use, dimethyl suberimide-HCl (DS) (Pierce, lot #081280-7) was dissolved in reaction buffer (30 mg/ml) and the pH quickly adjusted to pH 8.5. A one part aliquot of DS reagent was then added to nine parts of protein solution in the same buffer. The final DS and protein concentrations were 3 mg/ml and 160 ug/ml, respectively.

#### Termination of Chemical Cross-Linking Reactions

After incubating at 25°C for various time periods, individual reactions were terminated by addition of an excess of ethanolamine-HCl, pH 8.0. The final concentration of reaction quenching agent was 0.14 M for the DS cross-linking reaction. Twenty minutes later, the sample was made 1% in SDS and 2% in  $\beta$ -mercaptoethanol (total sample



volume = 1.0 ml). At the end of the time course, the samples (5-15 X 1.0 ml) were dialyzed against 1.5 l of 0.01 M sodium phosphate pH 7.1 containing 0.1% SDS, 0.1%  $\beta$ -mercaptoethanol, and 10% glycerol, overnight at 37°C. Each sample was then concentrated to a minimum volume ( ~ 25 ul) in an Amicon disposable multiwell CS-15 concentrator prior to gel application and electrophoresis.

#### Electrophoresis of Chemically Cross-Linked Proteins

SDS gel electrophoresis in a continuous buffer system was performed according to the procedure of Maizel (1966) as described by Shapiro et al. (1967), with modifications. To improve the resolution of higher molecular weight species, a final acrylamide gel composition of T = 3.8%, C = 2.63% (Bis), 0.1 M sodium phosphate, 0.1% SDS, pH 7.1 was selected. The electrophoresis buffer was 0.1 M sodium phosphate, 0.1% SDS, pH 7.1. The volume of the sample placed in the well (6 mm width) of the continuous slab gel was 10-20 ul. The dimensions of the separating gel were 13.5 X 14 X 0.15 cm. Cross-linked BSA (Sigma) was used as the  $M_r$  marker system ( ~ 15 ug / lane).

Electrophoresis was carried out under constant voltage at 50 volts initially for 0.5 hours, followed by 100 volts (14 watts power) for an additional 5.0 hours. Gels were stained, diffusion destained, and preserved as previously described.

#### Analytical Ultracentrifugation

Sedimentation velocity was determined with a Beckman Model E analytical ultracentrifuge using an AN-D rotor. Samples (4-7 mg/ml) in 0.1 M KCl, 0.1 mM DTE, 50 mM Tris-HCl pH 7.6 were run in a double

sector synthetic boundary capillary type cell (Beckman #306075) at 52,640 RPM and 20°C. Sample boundary movement was measured using Schlieren optics (bar angle 40-55°) and recorded on Kodak metallographic plates. Sedimentation coefficient values were corrected to those which would be obtained in water.

### Spectra

UV-visible spectra were obtained using a Beckman DU-8 spectrophotometer equipped with a  $\lambda$  scan compuset (series G) and 1 cm path-length cuvettes. Typical run conditions were as follows: scan rate = 50 nm/min, slit width = 0.2, and read average = 5. Cuvette temperature was maintained at 11-12°C by a piezoelectric temperature regulator which was part of the cuvette train assembly.

Prior to analysis samples were centrifuged in an Eppendorf model 5413 centrifuge (6500 X g, 3 minutes) to remove particulate matter. Molar extinction coefficients were calculated using a monomeric molecular weight of 84,000 for M1 and 58,000 for M2.

### Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance (EPR) first derivative spectra were recorded using either a Varian E-4 or an E-9 spectrometer both equipped with 100 kHz field modulation. Temperature control was achieved by using a quartz liquid nitrogen Dewar insert containing liquid nitrogen for those experiments at 77 K. In some cases, bumping due to liquid nitrogen boiling was minimized by coating the quartz EPR tube with glycerol prior to freezing by liquid nitrogen immersion (Chasteen, 1977). Samples were evaluated as prepared or with added

components in a 0.1 M KCl,, 0.1 mM DTE, 50 mM Tris-HCl pH 7.6 buffer.

A Minc computer (Digital Equipment Corp.) was attached on line to the Varian E-9 spectrometer. Software used for spectra accumulation and subsequent signal averaging (to improve the signal/noise ratio) were as described (Rosenberg, 1982).

#### IV. RESULTS

##### Purification of Calf Thymus Ribonucleotide Reductase

The purification of calf thymus ribonucleotide reductase was based upon modifications of a method initially outlined by Eriksson et al. (1977) and described in detail by Engström et al. (1979). The procedure was scaled up to accommodate 1.5-1.8 kg of calf thymus tissue as starting material. Table IV, a composite of three different enzyme preparations, shows typical results. The initial specific activity of the homogenate is in the area of that previously observed under the same assay conditions (Plumer, 1980). Streptomycin sulfate precipitation results in a substantial increase in the total number of enzyme units probably due to the precipitation of nucleic acids which are inhibitors of the enzyme. In the initial steps of purification, there are probably inhibitory factors present, therefore the specific activity values are probably not a good indicator of true enzyme content of fractions (Engström et al., 1979).

The stepwise elution of the DEAE-cellulose column (Plumer, 1980) facilitated the collection of fractions containing ribonucleotide reductase. The total yield of enzyme activity in the pools designated A through D varied from 50-100 units in successful operations. Those fractions containing enzyme with the highest specific activity (B,C) represent a 168-fold purification over the starting material. After adaptation of the scaled-up purification procedure, the assay of ribonucleotide reductase was not done until the DEAE-cellulose column

TABLE IV.

## Purification of Calf Thymus Ribonucleotide Reductase

Purification Step	Volume (ml)	Units ml	Total Activity (units)	[Protein] (mg/ml)	Specific Activity (units/mg)	Step-fold Purification	Percent Yield (stepwise)
1. Homogenate of 1.5 kg tissue	2700	0.036	97.2	19.2	0.0019	1.0	100
2. Supernatant from streptomycin precipitation	3480	0.014	487.2	14.3	0.0094	4.9	501
3. Resuspended, dialyzed ammonium sulfate precipitate	590	0.092	54.28	10.6	0.0086	0.91	11
4. DEAE-cellulose (column I) 0.1 M KCl pools	A	17.3	1.3	21.5	21.5	0.060	6.98
	B	13.8	3.0	41.4	8.86	0.33	38.37
	C	12.6	2.7	34.0	8.68	0.31	36.05
	D	12.5	0.76	9.5	6.59	0.11	12.79
5. dATP-Sepharose	1.15	38.0	43.7	6.68	5.7	17.81	58

fractions were collected. Only those preparations with enzyme activity yields as described above were used in subsequent purification steps.

Omission of the hydroxylapatite column procedure and replacing the gel chromatography desalting step (Engström et al., 1979) with dialysis simplified the purification scheme and allowed for the direct application of selected DEAE-cellulose column pools onto the dATP-sepharose affinity column. Application of 50-75 units of ribonucleotide reductase from the DEAE-cellulose column step to the affinity column generally yields 4-7 mg of protein with a specific activity of approximately 6 units/mg, corresponding to a purification of 15-20 for this step and an overall fold purification of approximately 3000.

Highly purified calf thymus ribonucleotide reductase preparations contain a major protein band,  $M_r \sim 84,000$ , and minor protein band,  $M_r \sim 58,000$ , corresponding to the M1 and M2 subunits respectively, in SDS-polyacrylamide gel electrophoresis (Figure 12). This finding is consistent with the previously reported occurrence of small non-stoichiometric quantities of the M2 subunit in the intact enzyme (Engström et al., 1979). In addition, other minor contaminants are also observed in the lower molecular weight region of the gel. Sample lane 4 in Figure 12 was purposefully overloaded to demonstrate the protein M1/M2 non-stoichiometry of the calf thymus ribonucleotide reductase preparation. A densitometric scan of the gel (Figure 13) indicates that the protein M1/M2 ratio is approximately 7/1 for an enzyme preparation with a specific activity of 5.8 units/mg.

Figure 12. SDS-polyacrylamide gel electrophoresis of calf thymus ribonucleotide reductase. Standards (1 and 5) are, from top: phosphorylase b ( $M_r = 94,000$ ), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and  $\alpha$ -lactalbumin (14,400). Ribonucleotide reductase (specific activity = 5.8 U/mg); 5, 12.5, and 25 ug in lanes 2, 3, and 4, respectively.

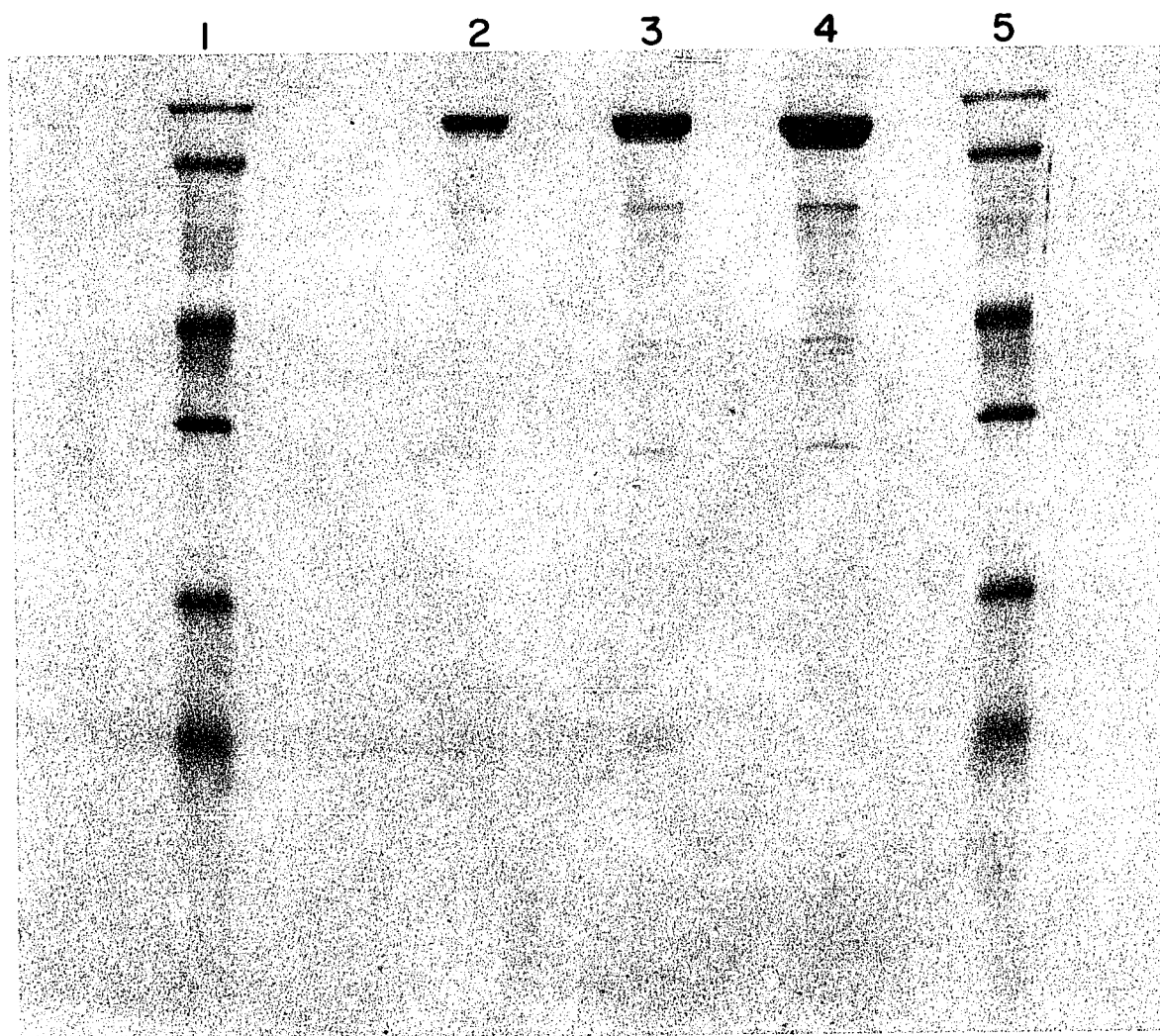
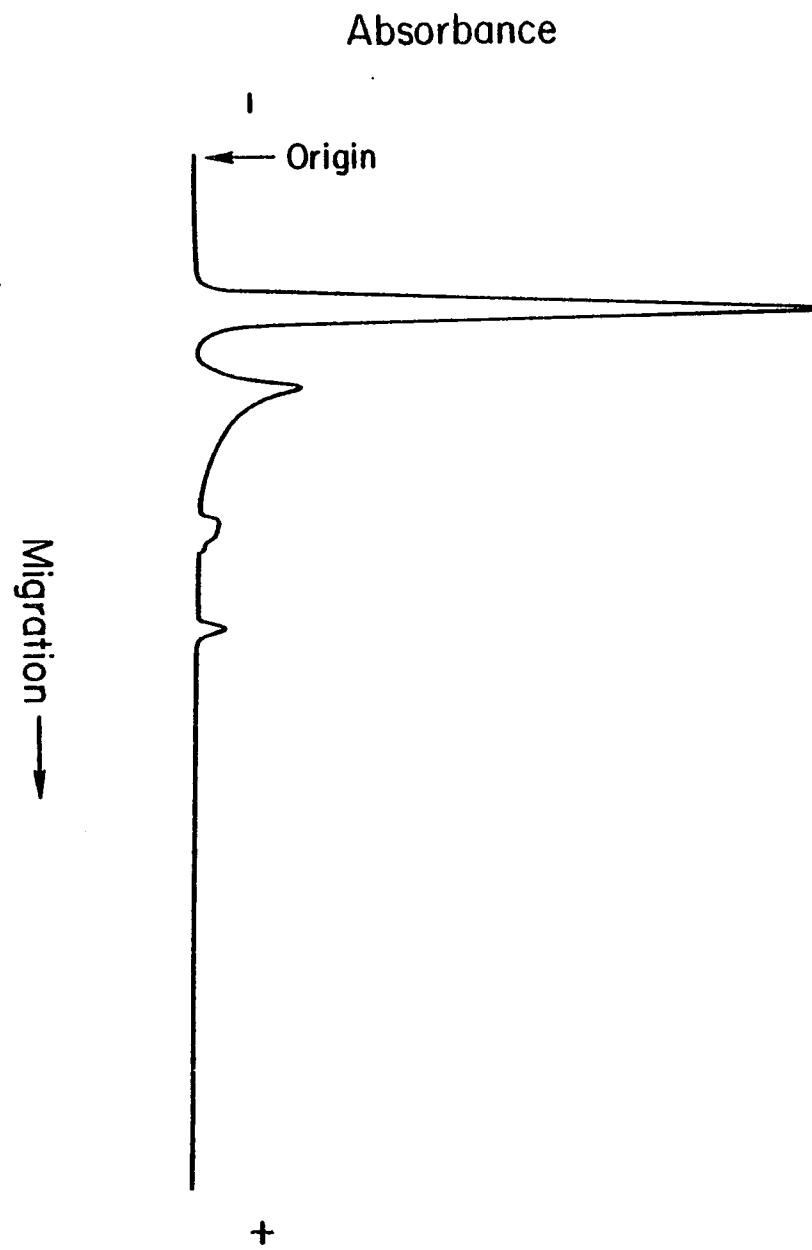




Figure 13. Densitometric scan of ribonucleotide reductase on an SDS-polyacrylamide gel. (lane 3, Figure 12).



### Separation of the Complementary Subunits

The final purification step achieves the separation of the M1 and M2 subunits of the ribonucleotide reductase. Chromatography of the enzyme on a DEAE-cellulose column in the presence of 4 mM ATP and 4mM  $Mg^{++}$  results in the dissociation of the two complementary subunits (Plumer, 1980). Their separation is a result of binding of allosteric effector, ATP, to the M1 subunit so that a higher concentration of KCl (0.25 M) is required for the M1 subunit elution than that needed in the absence of ATP or than that needed for protein M2 elution (0.1 M KCl). A representative elution profile for a DEAE-cellulose column used in this step is shown in Figure 14. Eluant absorbance was monitored at 295 nm. Detection of protein elution by monitoring the absorbance at 280 nm is prevented by the presence of 4 mM ATP in the column buffer.

The purification of the M1 and M2 subunits of calf thymus ribonucleotide reductase are summarized in Table V. Protein M1 and M2 activity were determined by assay in the presence of an excess of the complementary subunit. Protein M1 activity was determined in the presence of partially purified M2 prepared according to Plumer (1980). Protein M2 activity was determined by assay in the presence of highly purified M1 subunit, obtained as described above. The specific activity of protein M1 differs from preparation to preparation. This variation may be related, in part, to inhibition of ribonucleotide reductase M1 by excess protein M2, when the complementary subunits are re-mixed for assay (Thelander et al., 1980). However, the weight of protein M1 recovered is always considerably larger than that of pro-

Figure 14. Elution profile of the complementary subunits of ribonucleotide reductase from DEAE-cellulose (column II) in the presence of 4 mM ATP and 4 mM  $\text{Mg}^{2+}$ . Conditions are as described in Materials and Methods.

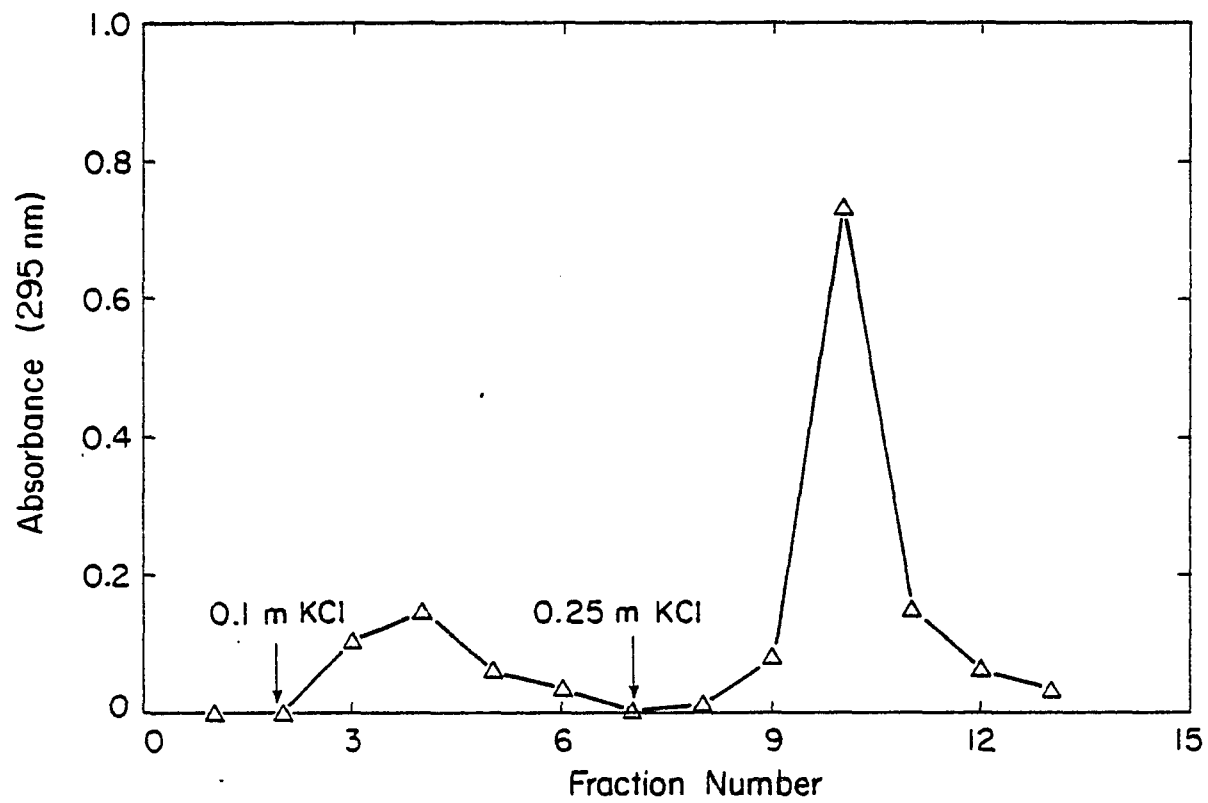


TABLE V.

## Purification of M1 and M2 Subunits of Ribonucleotide Reductase from Calf Thymus

Purification step	Volume (ml)	Activity Intact	(Units)* M1	M2	[Protein] (mg/ml)	Specific Activity (units/mg)	Percent Yield (stepwise)
Intact ribonucleotide reductase	1.22	59.5	0		5.58	8.73	-
DEAE-cellulose column (4 mM in ATP and Mg <sup>2+</sup> )							
0.1 M KCl eluate	0.76	0		4.71	0.75	8.46	9
0.25 M KCl eluate	0.96	3.14	10.2		4.02	2.64	22

\*M1 and M2 activity determined in the presence of an excess of the complementary subunit.

tein M2. This finding is in agreement with the previously reported occurrence of small non-stoichiometric amounts of protein M2 in intact enzyme (Engström et al., 1979).

The purified M1 and M2 subunit preparations contain single proteins when analyzed by SDS-polyacrylamide gel electrophoresis (Figure 15). The polypeptide molecular weight ( $M_r$ ) of M1, obtained from an SDS-polyacrylamide gel run according to the method of Weber and Osborn (1969) is 84,000 (Figure 16). A discontinuous buffer SDS-gel electrophoresis system (Laemmli, 1970) was selected for the M2 polypeptide molecular weight determination due to its improved resolving power and lack of zone spreading in the molecular weight region of interest (30-65 K). The  $M_r$  of the M2 polypeptide is 58,000 (Figure 17).

#### Complementary Subunit Physical Composition

##### Amino Acid Analysis

The amino acid composition data for the M1 and M2 subunits of calf thymus ribonucleotide reductase is given in Table VI. Examination of the ratio of hydrophilic and hydrophobic amino acids, ( $\text{Ratio}_3 = \text{Lys} + \text{Arg} + \text{His} + \text{Asx} + \text{Glx} / \text{Ile} + \text{Tyr} + \text{Phe} + \text{Leu} + \text{Val} + \text{Met}$ ) (Barrantes, 1975), reveals  $R_3$  values of 1.086 and 1.300 for the M1 and M2 subunits, respectively. These  $R_3$  values are within the range of those values observed for numerous other soluble, or peripheral but not integral membrane proteins (Barrantes, 1975). A direct comparison of the calf thymus and E. coli ribonucleotide reductase complementary subunit amino acid composition can be seen in Table VII. The data indicates compositional homology between proteins M1 and B1 ( $R_3 =$

Figure 15. SDS-polyacrylamide gel electrophoresis of the complementary subunits of calf thymus ribonucleotide reductase: Standards as in Figure 12 (1 and 6). Subunits: M1 (2 and 3) 10 ug/lane, specific activity: 3.83 U/mg; M2 (4 and 5) 5 ug/lane, specific activity: 4.32 U/mg.



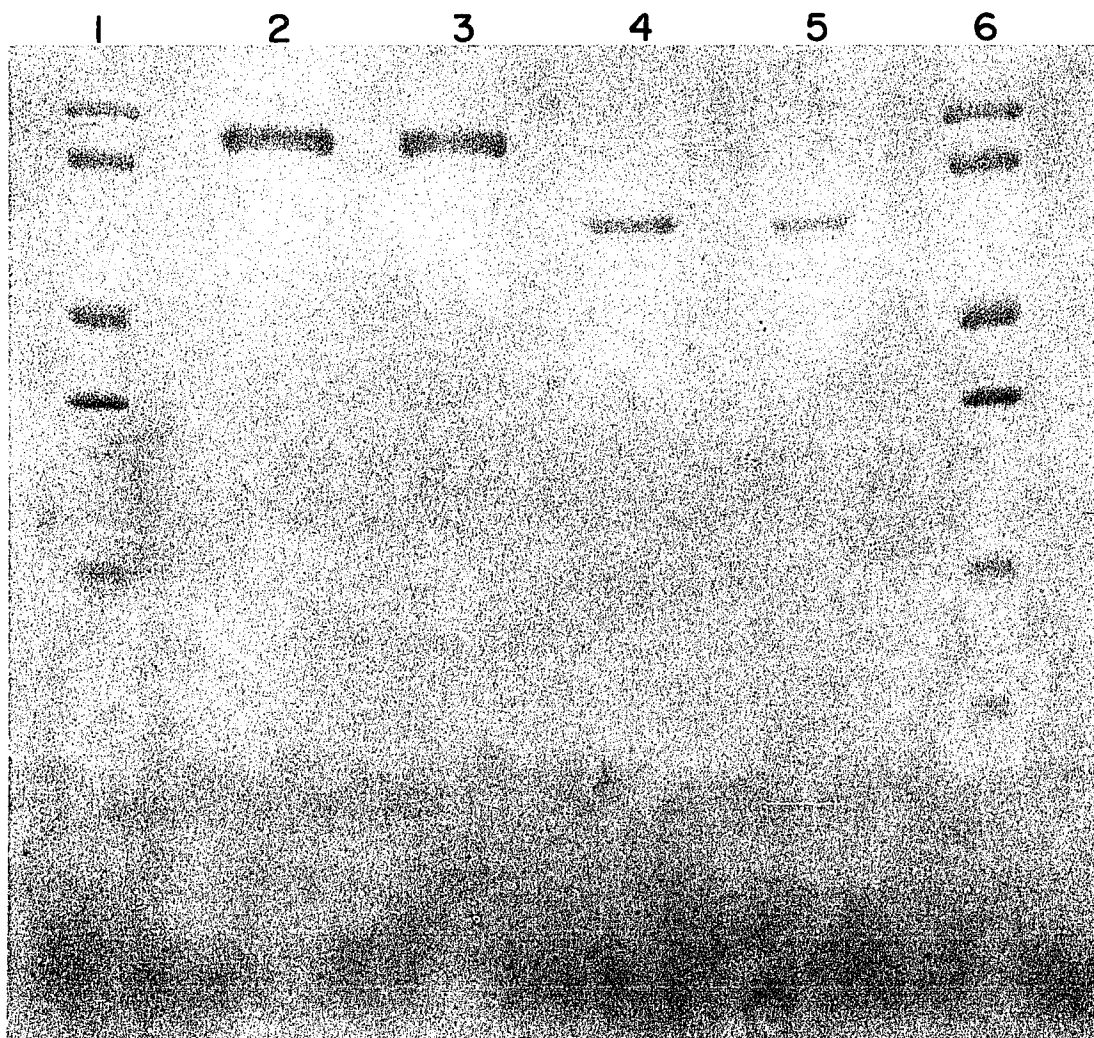


Figure 16. Calibration curve for SDS-polyacrylamide gel electrophoresis in 10% acrylamide gels run according to the method of Weber and Osborn (1969). Protein standards include: 1. phosphorylase b, 2. bovine serum albumin, 3. ovalbumin, 4. carbonic anhydrase, and 5, trypsin inhibitor and 6.  $\alpha$ -lactalbumin. The  $R_f$  of protein M1 is indicated by the arrow.

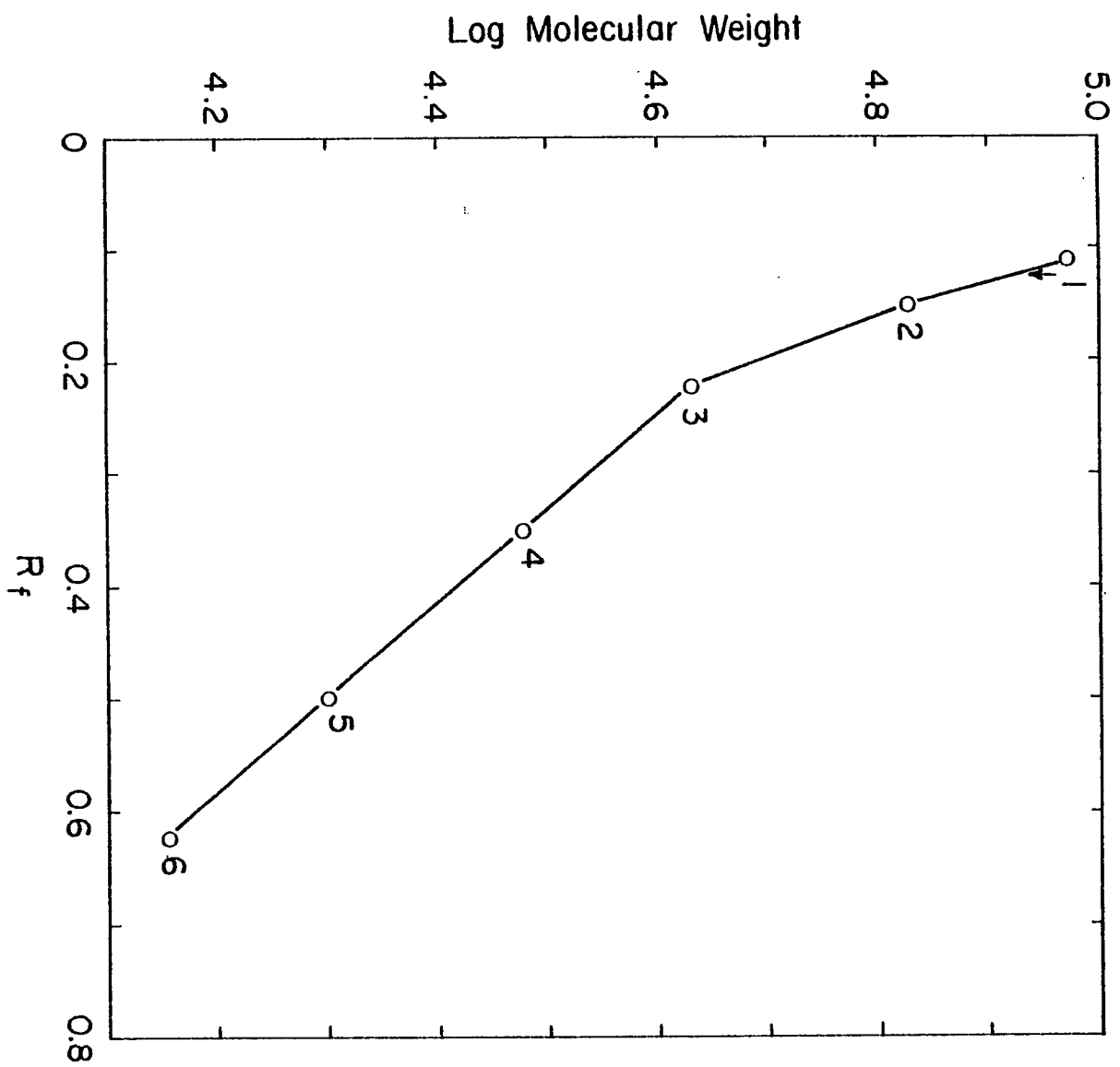


Figure 17. Calibration curve for the 11-14% exponential gradient SDS-polyacrylamide gel. Protein standards are as in Figure 16. The  $R_f$  of protein M2 is indicated by the arrow.

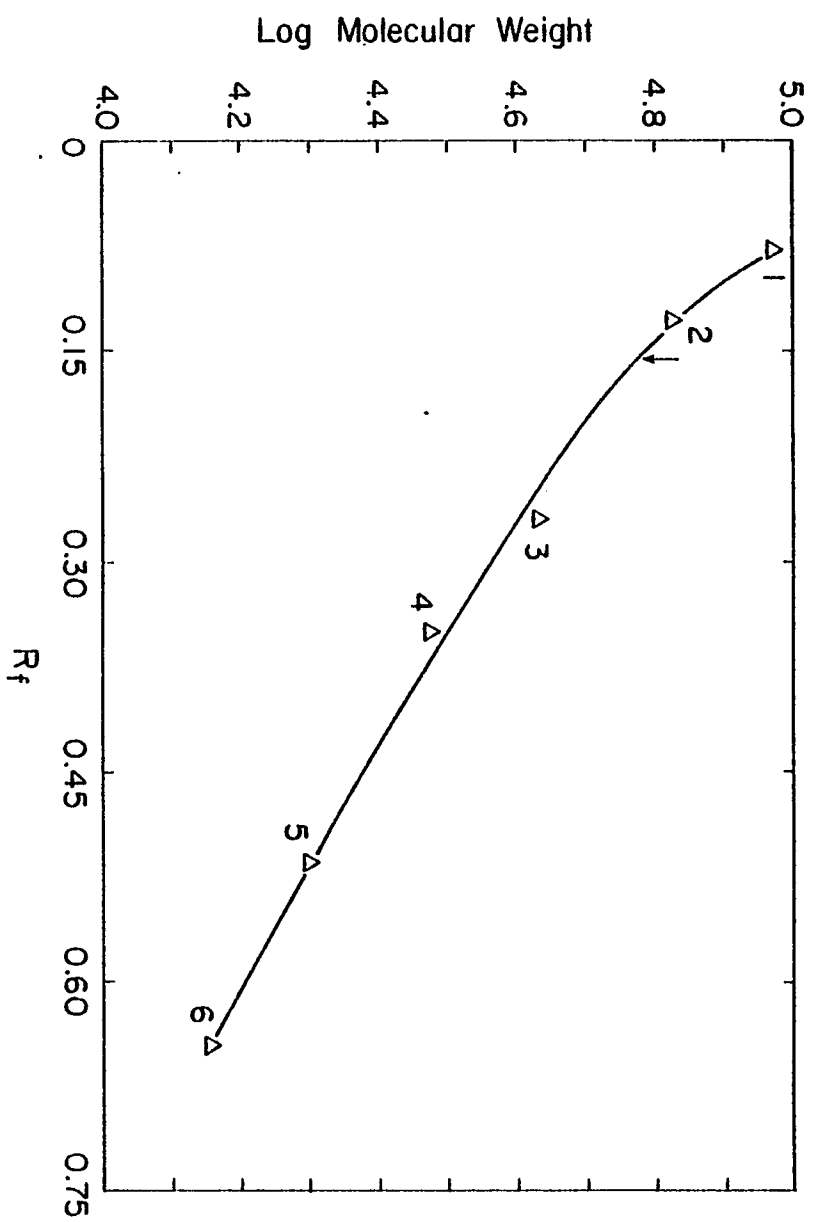


TABLE VI.

The Amino Acid Composition of Purified Calf Thymus Ribonucleotide  
Reductase Subunits M1 and M2

<u>Amino Acid Residue</u>	<u>Residues/Mole Monomer</u>			
	M1 (regulatory)		M2 (catalytic)	
	subunit		subunit	
		nearest integer		nearest integer
Aspartic acid	80.8	81	55.0	55
Threonine*	43.7	44	23.9	24
Serine*	44.6	45	33.9	34
Glutamic acid	81.4	81	54.5	55
Proline	30.2	30	27.9	28
Glycine	43.9	44	57.0	57
Alanine	49.9	50	32.7	33
Half-cystine <sup>+</sup>	12.4	12	6.9	7
Valine	40.3	40	30.5	31
Methionine	18.7	19	6.0	6
Isoleucine <sup>Δ</sup>	47.5	48	25.0	25
Leucine <sup>Δ</sup>	74.0	74	41.9	42
Tyrosine	35.0	35	14.7	15
Phenylalanine	26.0	26	23.0	23
Histidine	15.8	16	16.0	16
Lysine	53.1	53	31.1	31
Tryptophan <sup>#</sup>	7.2	7	4.0	4
Arginine	31.2	31	26.8	27

\*Extrapolated to zero time to correct for losses during hydrolysis.

<sup>+</sup>Determined after oxidation with performic acid.

<sup>Δ</sup>Extrapolated to infinite hydrolysis time.

<sup>#</sup>Determined after hydrolysis using 4 N methanesulfonic acid containing 0.2% 3(2-aminoethyl)indole.

TABLE VII.

A Comparison of the Amino Acid Composition Data for Ribonucleotide  
Reductase Subunits from Calf Thymus (M Type) and E. coli (B Type)

<u>Amino Acid Residue</u>	<u>Residues/100 Residues</u>			
	<u>Regulatory Subunits</u>		<u>Catalytic Subunits</u>	
	M1	B1 <sup>*</sup>	M2	B2 <sup>*</sup>
Aspartic acid	11.0	11.2	10.8	10.7
Threonine	5.9	4.8	4.7	5.1
Serine	6.1	6.1	6.6	6.0
Glutamic acid	11.1	10.7	10.7	14.3
Proline	4.1	4.4	5.5	3.9
Glycine	6.0	6.4	11.2	4.7
Alanine	6.8	8.3	6.4	7.3
Half-cystine	1.7	1.5	1.4	1.4
Valine	5.5	5.2	6.0	6.8
Methionine	2.5	1.9	1.2	2.1
Isoleucine	6.5	6.5	4.9	6.8
Leucine	10.1	9.9	8.2	9.8
Tyrosine	4.8	5.2	2.9	3.7
Phenylalanine	3.5	3.7	4.5	4.3
Histidine	2.1	2.5	3.1	2.1
Lysine	7.2	5.6	6.1	4.3
Tryptophan	1.0	0.7	0.8	1.7
Arginine	4.2	5.2	5.2	4.9
Subunit molecular weight	84,000	160,000 (dimer)	58,000	78,000 (dimer)

<sup>\*</sup>(from Thelander, 1973)

1.091), but not M2 and B2 ( $R_3 = 1.076$ ). The correlation coefficients for the comparison of the calf thymus vs. E. coli regulatory and catalytic subunits are 0.972 and 0.801, respectively. Graphical representation of the compositional comparison for each subunit type from calf thymus and E. coli ribonucleotide reductase are presented in Figures 18 and 19 (Cantor and Schimmel, 1980). A quantitative evaluation of the degree of compositional homology between the regulatory and catalytic subunits from the mammalian and bacterial sources was made according to the method of Marchalonis and Weltman (1971). The results of this calculation and a definition of the method of comparison,  $S \Delta Q$ , are given in Table VIII. Comparison of the M1 and B1 subunit types yields an  $S \Delta Q$  value of 8.9. This value satisfies the criteria for a "strong" test of similarity which indicates that the two regulatory subunit types are related (Cornish-Bowden, 1981). The analogous result for a comparison of the catalytic subunit types ( $S \Delta Q = 72.7$ ) satisfies neither the criteria for a "strong" test nor that for a "weak" test of similarity, a less reliable indicator of protein relatedness. However, it should be noted that a significant difference in the polypeptide molecular weights of proteins M2 (58,000) and B2 (39,000) occurs. Application of mathematical methods for compositional comparison to proteins of significantly different size may yield results which are subject to significant error and difficult to interpret.

#### Analytical Peptide Mapping

The amino acid composition data for the regulatory subunits of calf thymus and E. coli ribonucleotide reductase indicates that significant compositional, and presumably structural, homology exists



Figure 18: Comparison of the composition data for the regulatory subunit of ribonucleotide reductase from calf thymus (M1) and E. coli (B1, shaded areas). The mole fraction of each amino acid is plotted in approximate order of decreasing hydrophobicity.

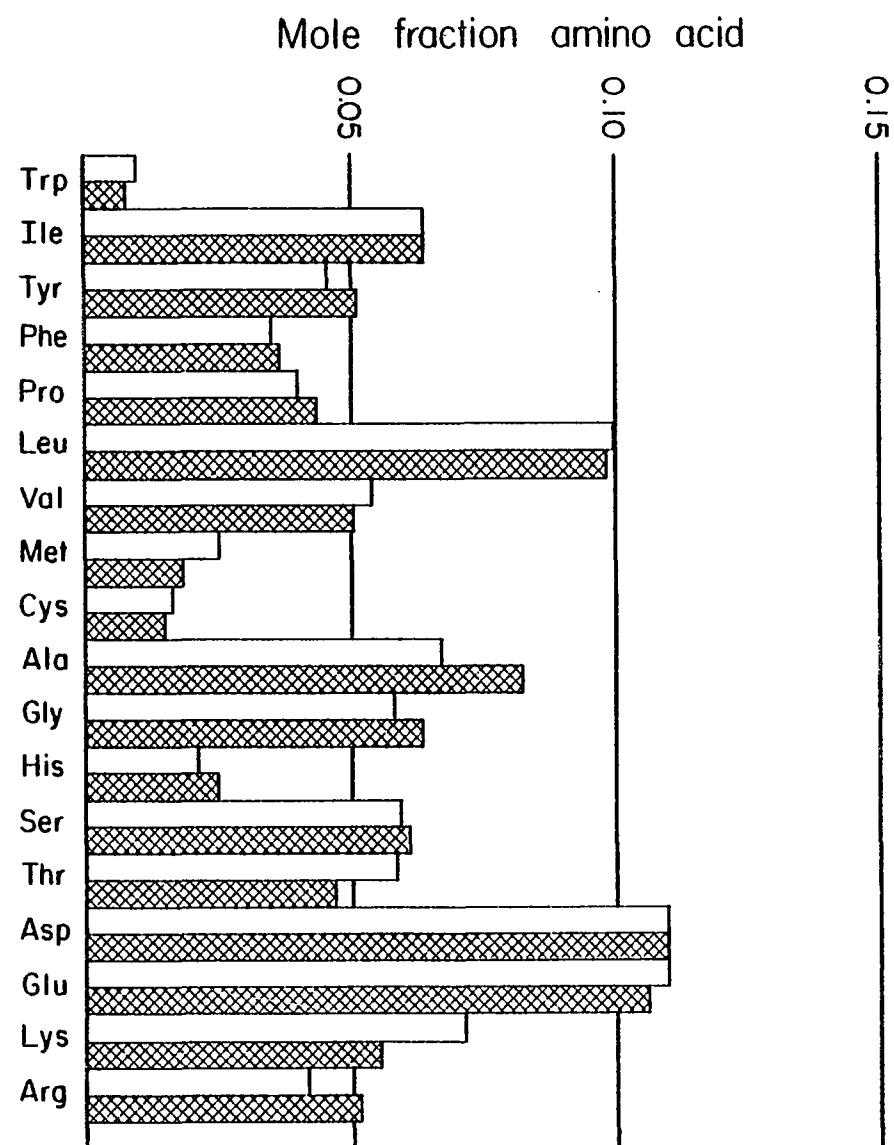


Figure 19. Comparison of the composition data for the catalytic subunit of ribonucleotide reductase from calf thymus (M2) and E. coli (B2, shaded areas). Data is presented as in Figure 18.

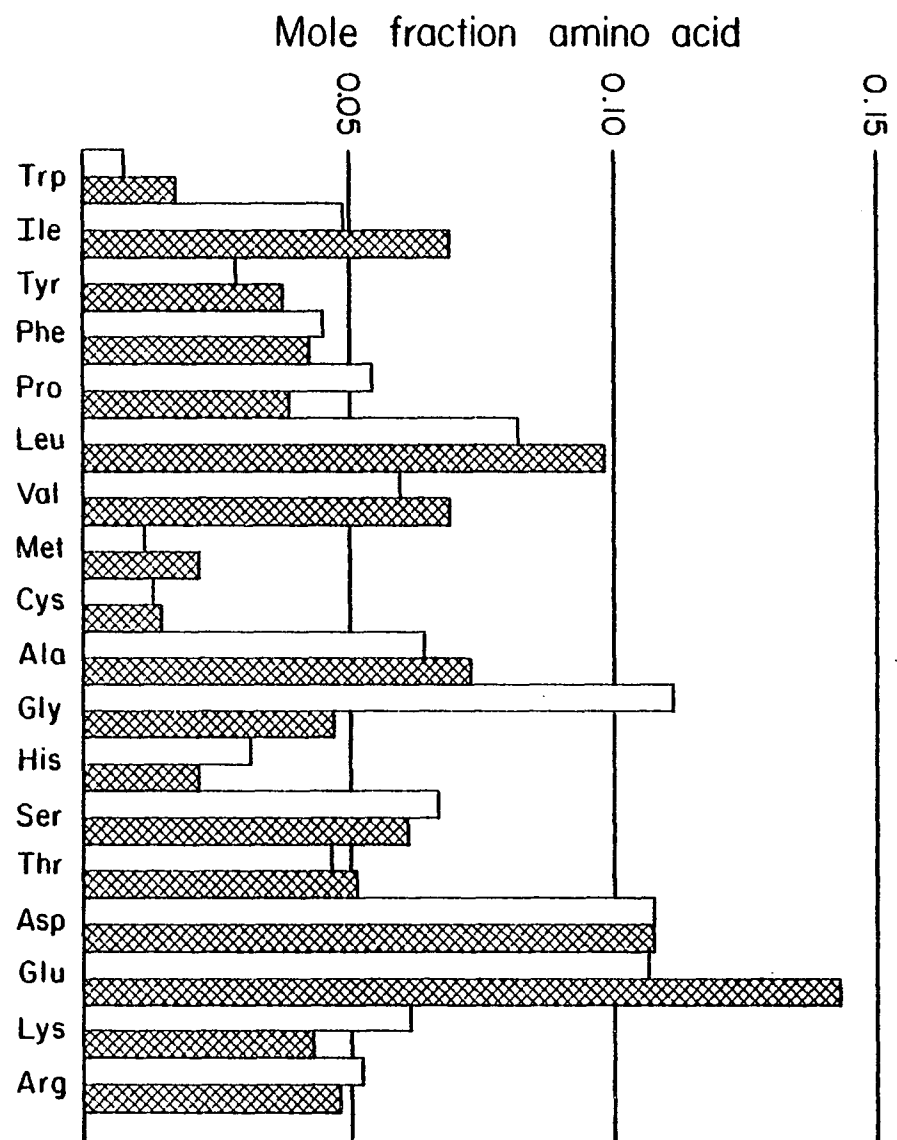


TABLE VIII.

An Estimate of Ribonucleotide Reductase Subunit Relatedness

SAQ\* (Calf Thymus compared to E. coli)

	M1	M2
B1	8.9	40.2
B2	26.4	72.7

\* Comparative method used is based on amino acid composition:

$$SAQ^* \equiv \sum_j (X_{i,j} - X_{k,j})^2$$

i,k - identify the particular proteins being compared

$X_j$  - content of amino acid type j expressed as residues/100 residues

\* Marchalonis, J.J., and Weltman, J.K. (1971)

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between the two polypeptides. To further investigate this possibility, comparative peptide mapping of calf thymus protein M1 and E. coli protein B1 was performed. Highly purified samples of each regulatory subunit were partially digested with Staphylococcus aureus (V8) protease in the presence of SDS according to Cleveland et al. (1977). The resultant peptide map of each protein is shown in Figure 20. In contrast to the suggestion from the amino acid composition data that the M1 and B1 polypeptides might be structurally similar, the comparative peptide maps reveals the existence of proteolytic degradation products unique to both subunits. Thus distinct differences in the primary structure of each protein are indicated. This information suggests that, although proteins M1 and B1 have similar functions, size, and composition, the regulatory subunits are structurally dissimilar.

#### M2 Subunit Iron Content Determination

The requirement of protein M2-associated non-heme iron (Plumer, 1980) for calf thymus ribonucleotide reductase activity has been reported (Engstrom et al., 1979; Thelander and Reichard, 1979). Direct analysis of the iron content of protein M2 is possible with the homogenous protein M2 preparation. The results of the atomic absorption spectroscopic determination of protein M2-associated iron are presented in Table IX. The results indicate that protein M2 contains approximately 3 g-atoms Fe/mole protein M2 (monomer). This is in contrast to the reported 1 g-atom Fe/mole protein B2 (monomer) in the E. coli ribonucleotide reductase (Brown et al., 1969a).

The function of the observed protein M2-associated iron is not

Figure 20. Comparative peptide map of calf thymus M1 and E. coli B1 subunit types. Lane 1, calf thymus M1; 4, E. coli B1; 2 and 3, M1 and B1, respectively, treated with S. aureus V8 protease as described under Materials and Methods. The arrows indicate the migration distances of molecular weight standards: from top, phosphorylase b ( $M_r = 94,000$ ) bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and  $\alpha$ -lactalbumin (14,400).



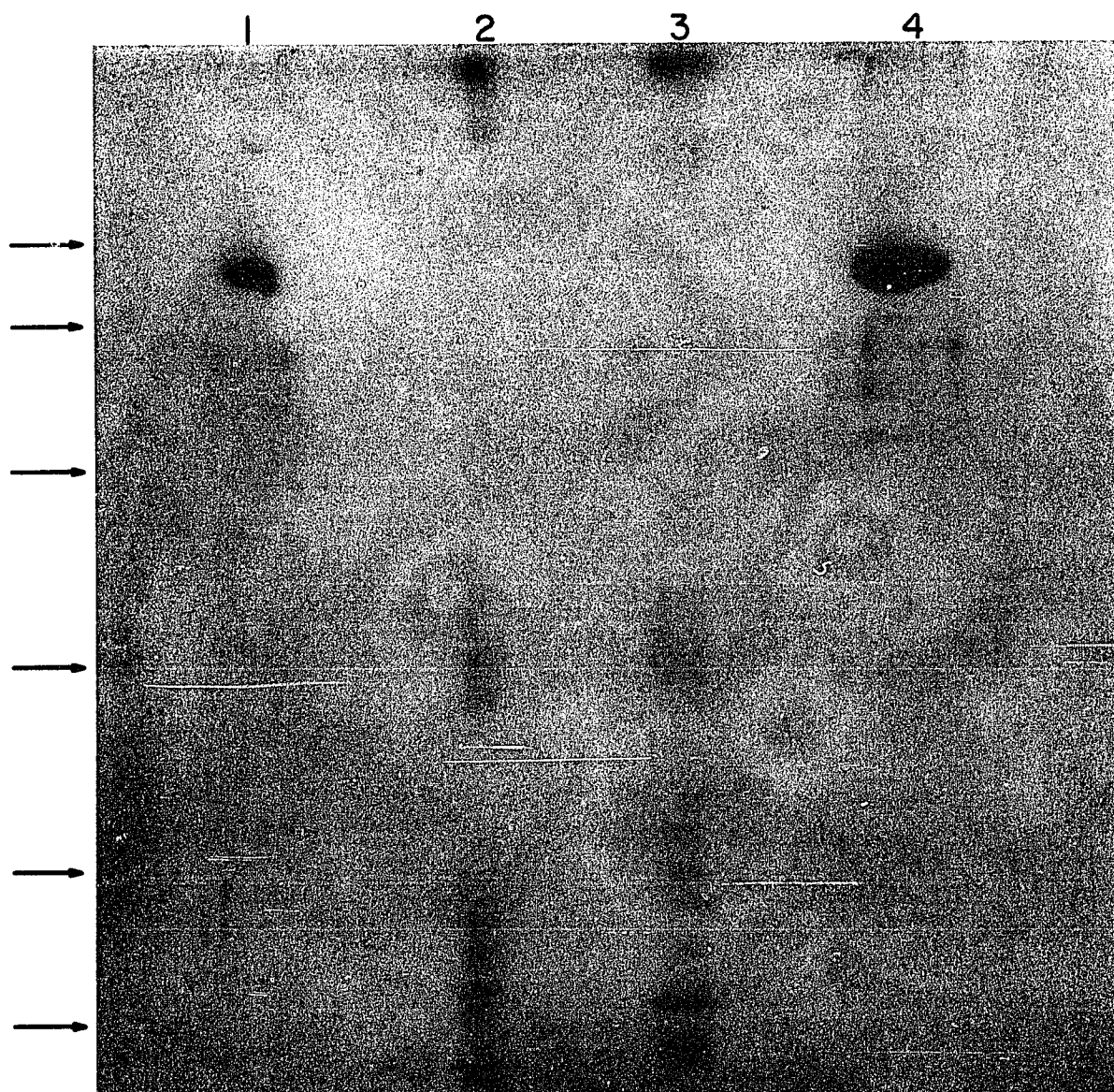


TABLE IX.

## Ribonucleotide Reductase M2 Subunit Iron Content Analysis

<u>Sample</u>	<u>Specific activity</u> <sup>*</sup>	<u>g-Atoms Fe/mole M2</u> <sup>b</sup>	
	units/mg <sup>a</sup>	Buffer System	
1	11.05	2.5	2.9
2	4.97	2.9	3.0

Sample treatment: purified M2 samples in 0.1 M KCl, 0.1 mM DTE, 50 mM Tris-Cl pH 7.6 (buffer A) were evaluated as prepared, and also buffer exchanged into Chelex-100 treated 0.1 M KCl, 0.1 mM DTE, 50 mM HEPES pH 7.6 (buffer B).

Protein determinations: a - Bradford, b - amino acid analysis.

\* Assayed in the presence of an excess of complementary subunit

clearly understood. Ribonucleotide reductase preparations have been reported to be stimulated by the addition of exogenous iron (Hopper, 1972, 1978; Moore, 1977). Iron is routinely added, as  $\text{FeCl}_3$ , to our assay mixtures. However, titration of constant amounts of highly purified calf thymus ribonucleotide reductase with iron as  $\text{FeCl}_3$  or as an  $\text{Fe(II)}$ ascorbate complex results in activation of the enzyme. The activation profile is shown in Figure 21. Both  $\text{FeCl}_3$  and  $\text{Fe(II)}$ -ascorbate activate ribonucleotide reductase to the same degree. The magnitude of change is typically an approximate 2-fold increase in enzyme activity in the presence of excess metal cofactor. Thus, the role of protein M2-associated iron and its interaction, if any, with exogenous iron remains to be clarified.

#### Ribonucleotide Reductase Complementary Subunit Solution Hydrodynamics Sedimentation Velocity Studies

The sedimentation behavior of highly purified calf thymus ribonucleotide reductase was evaluated using the analytical ultracentrifuge. The enzyme sediments as a single slightly assymmetric peak of 6.2 s (Figure 22). Addition of dATP and  $\text{Mg}(\text{CH}_3\text{COO}^-)_2$ , at final concentrations of 50  $\mu\text{M}$  and 10 mM respectively, to the enzyme induces a change in the sedimentation profile. In the presence of allosteric effector, a major (17.0 s) and a minor Schlieren peak (6.4 s) are observed (Figure 23). Calf thymus ribonucleotide reductase, prepared as previously described, is largely comprised (80-90%) of M1 subunit. In the absence of nucleoside triphosphate effectors, protein M1 behaves as a monomer with a sedimentation coefficient of 5.6-5.7 s (Plumer, 1980; Thelander et al., 1980). The results (Figure 22) indicate that intact enzyme, in the absence of allosteric effectors,

Figure 21. Activation of calf thymus ribonucleotide reductase by iron: triangles, Fe(II)ascorbate; circle, FeCl<sub>3</sub> (60 uM); square, 3.0 mM Na ascorbate control. Molar ratio of ascorbate to Fe in Fe(II) complex is 20:1 (Atkin et al., 1973).

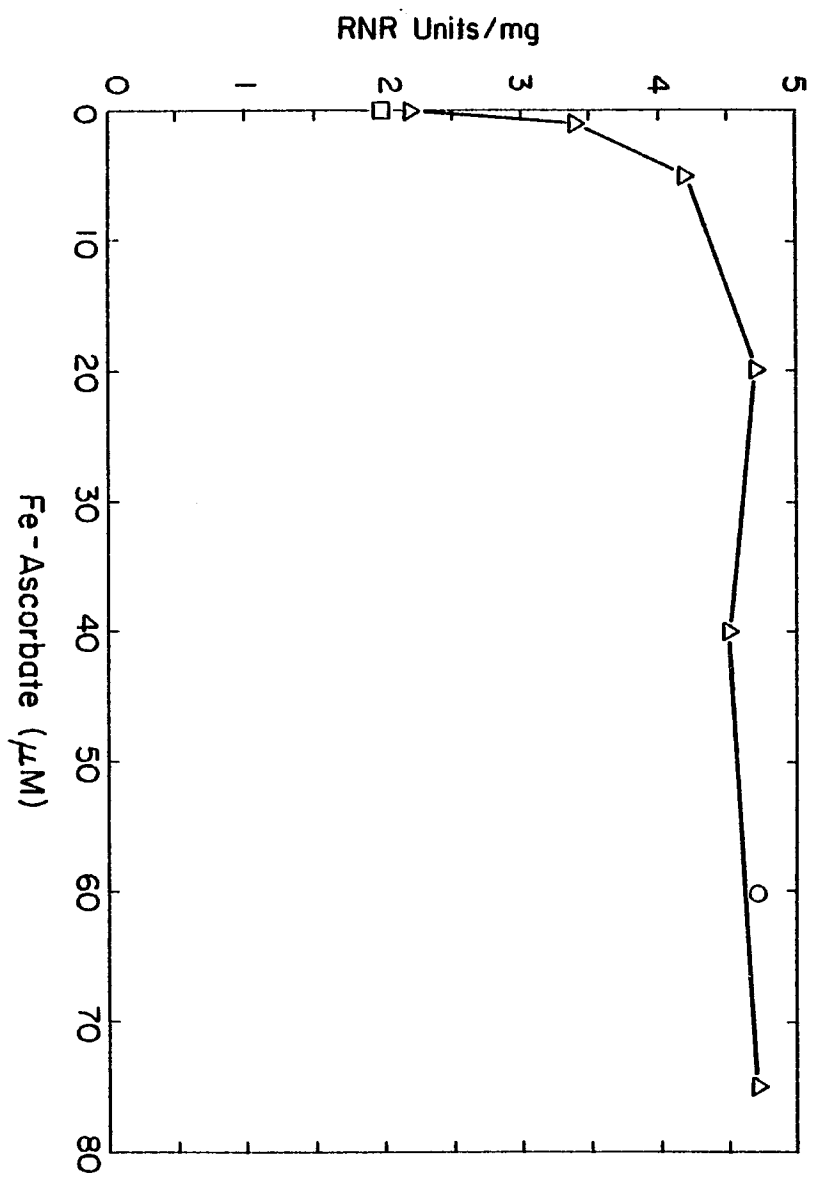


Figure 22. Determination of the sedimentation coefficient of non-dissociated calf thymus ribonucleotide reductase. Enzyme sample: specific activity - 5.7 U/mg; 6.5 mg/ml.

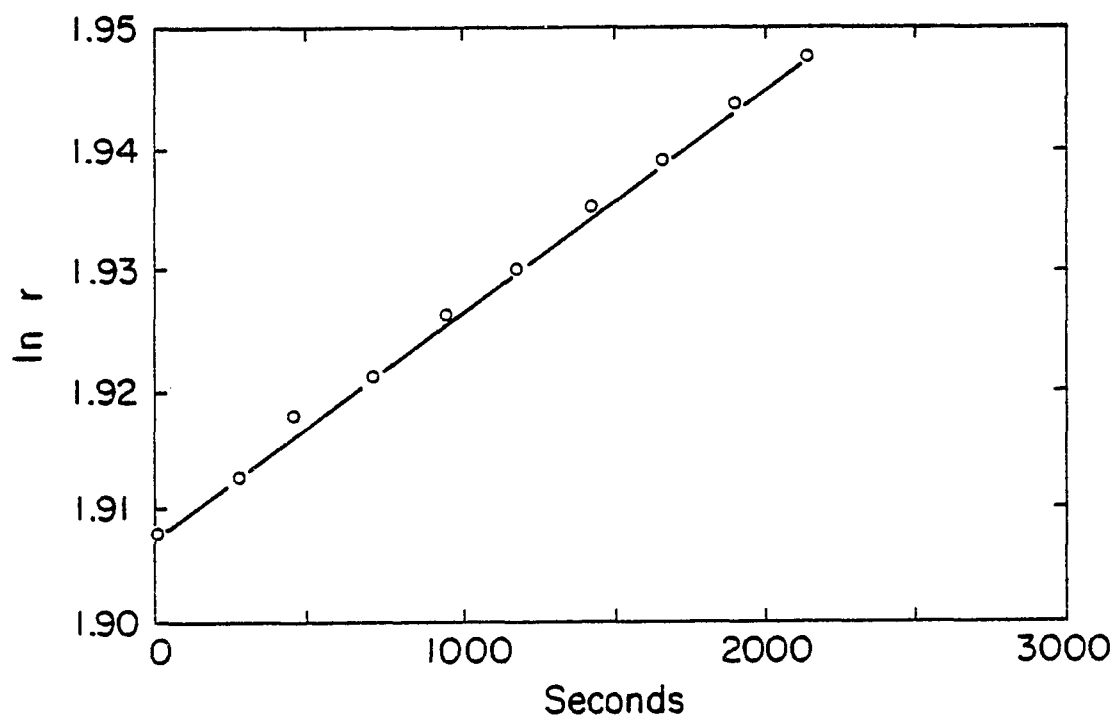
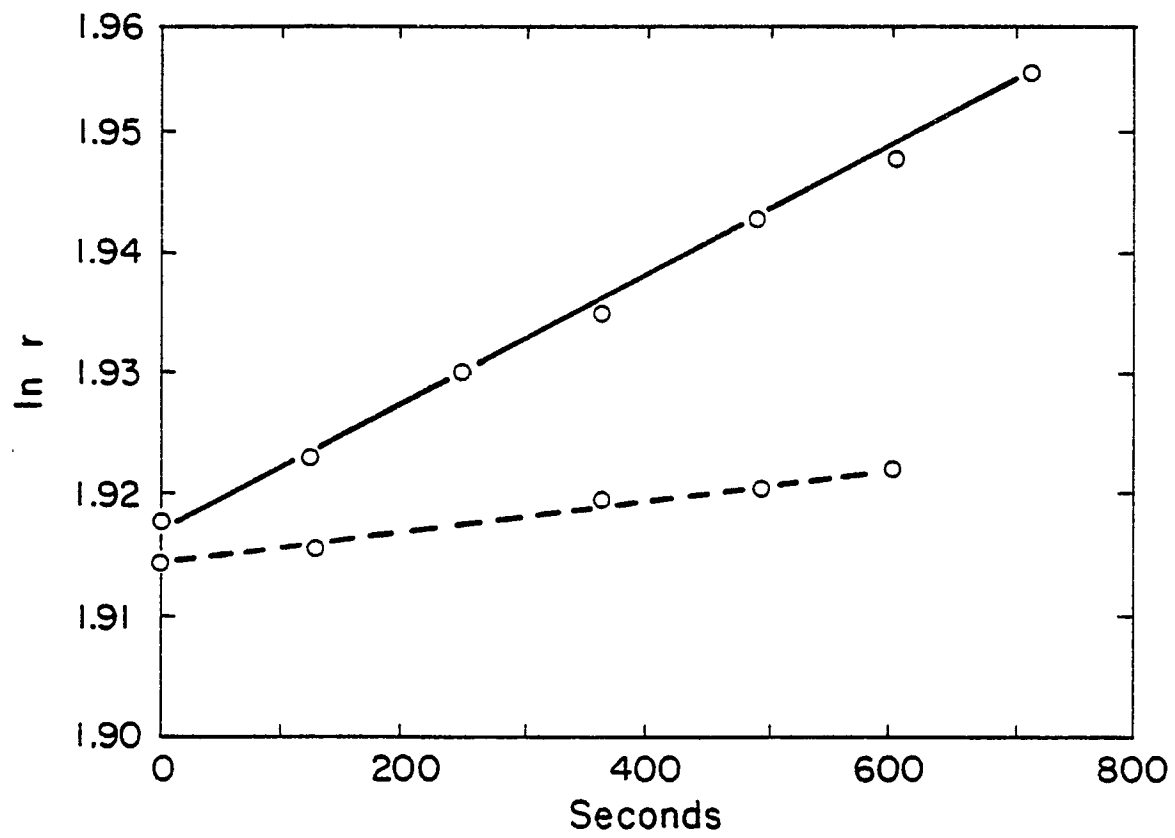


Figure 23. Determination of the sedimentation coefficient of calf thymus ribonucleotide reductase in the presence of 50  $\mu$ M dATP. Enzyme sample: specific activity - 2.1 U/mg, 7.8 mg/ml. (---) minor Schlieren peak.





moves in sedimentation as though a rapid mobile equilibrium exists between proteins M1 and M1M2 since the  $s_{20,w}$  is shifted upward to 6.2 s (from 5.7 s for M1). The presence of dATP (47  $\mu$ M) is reported to induce protein M1 tetramer (15.2 s) formation as observed in analytical glycerol gradient centrifugation (Thelander et al., 1980). Similar results have also been obtained using ribonucleotide reductase from Ehrlich tumor cells (Klippenstein and Cory, 1978). In addition,  $Mg^{2+}$  has no effect upon protein M1 sedimentation behavior (Thelander et al., 1980). The formation of a 17 s species (Figure 23) in the presence of dATP is most likely the result of protein M1 tetramer formation. The trailing 6.3 s peak also observed under these conditions is consistent with an M1M2 protein complex in equilibrium with protein M1 tetramer.

#### Analytical Liquid Gel Chromatography

Further evidence for calf thymus ribonucleotide reductase complementary subunit homogeneity was sought by examining the elution profiles of highly purified proteins M1 and M2 in analytical high performance liquid gel chromatography (Figure 24). Both M1 and M2 proteins ran as symmetrical peaks with  $K_d$ 's of 0.43 and 0.28, respectively, corresponding to Stoke's radii of 37 A and 53 A, respectively (Figure 25). A molecular weight plot (Figure 24) yields  $M_r = 70,000$  for protein M1 and 185,000 for protein M2. A small peak with a Stoke's radius of 54.5 A was also observed in the protein M1 chromatogram and is attributed to protein M1 dimer, since SDS-polyacrylamide gel electrophoresis indicated the protein M1 preparation to be homogenous. Thus, the M1 subunit is predominantly monomeric under experimental condition while the M2 subunit is

Figure 24. High performance liquid gel chromatography of native M1 and M2 subunits. A) Standard curve of ferritin (1),  $\gamma$ -globulin (2), aldolase (3), hexokinase (4), ovalbumin (5), and soybean trypsin inhibitor (6). B) M2 subunit (specific activity: 7.85 U/mg). C) M1 subunit (specific activity: 3.83 U/mg).

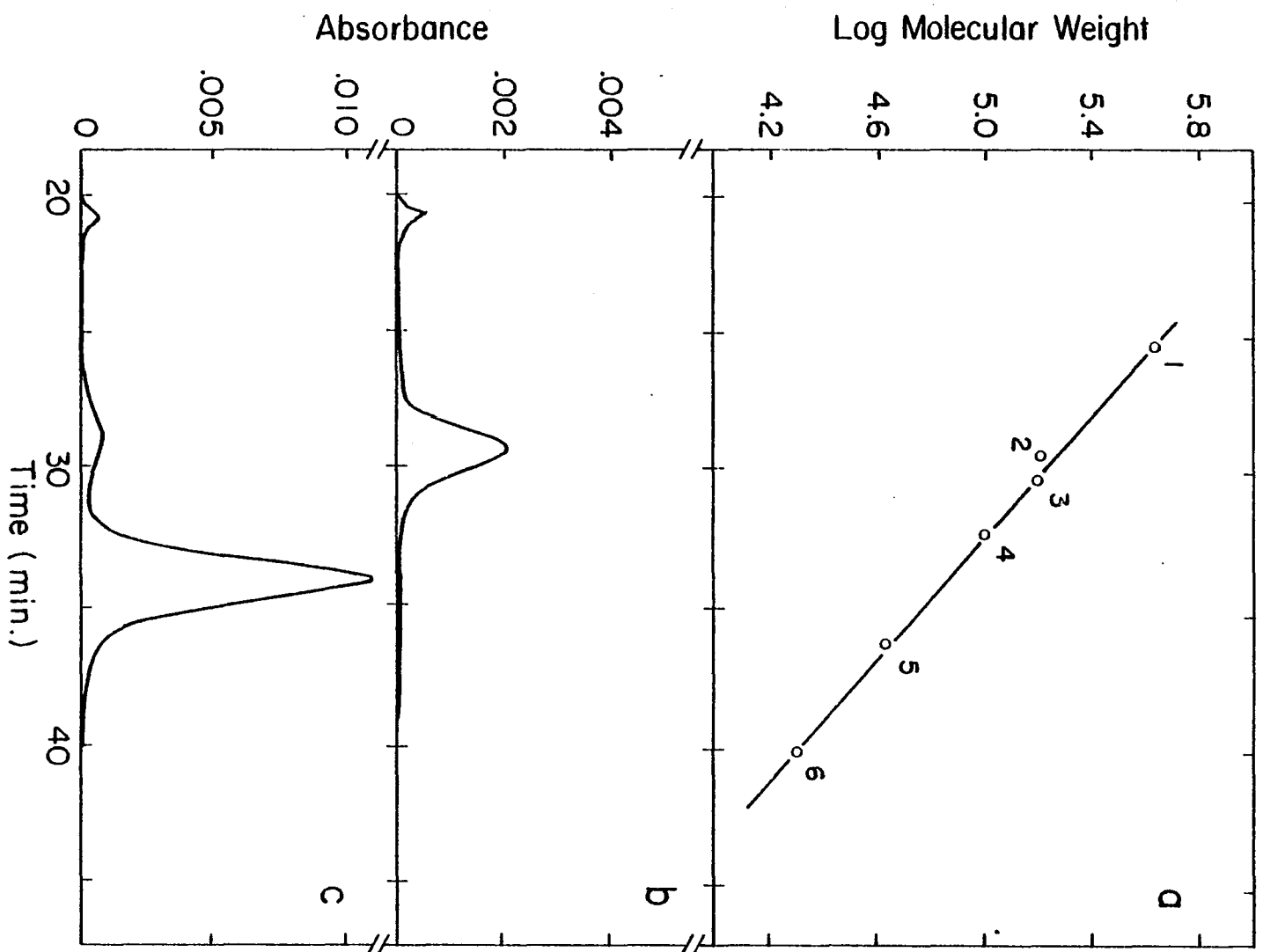
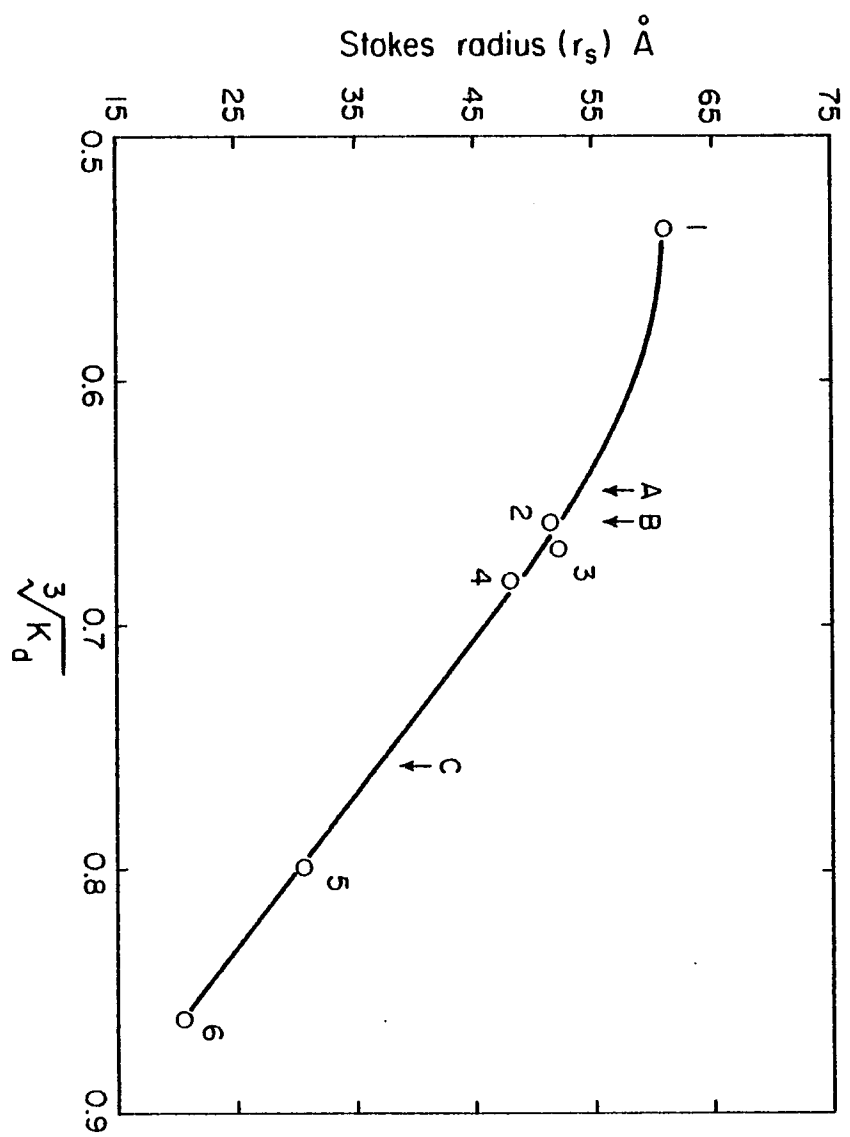


Figure 25. Determination of the Stoke's radii of the complementary subunits of calf thymus ribonucleotide reductase. Standard curve of ferritin (1),  $\gamma$ -globulin (2), catalase (30), aldolase (4), ovalbumin (5), and soybean trypsin inhibitor (6). Distribution coefficients indicated by arrows: A) M1 dimer, B) M2, C) M1.



oligomeric. Both the M1 and M2 protein elution profiles contained a minor component eluting in the void volume ( $M_r > 500,000$ ) which are probably aggregates formed during sample freezing and thawing.

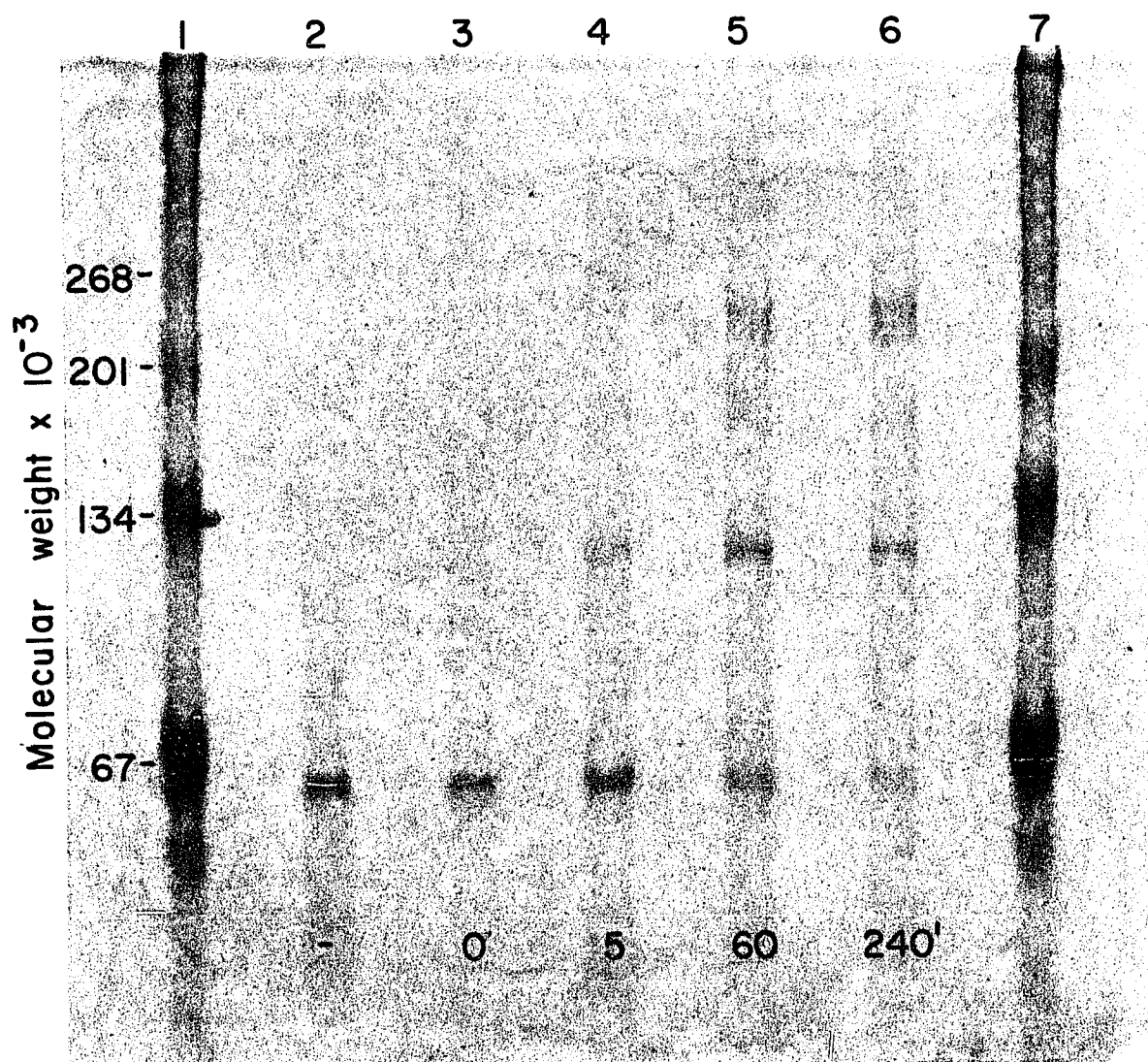
### Oligomeric Structure Determination

#### Ribonucleotide Reductase Catalytic Subunit

Native M2 subunit behaves as a 185,000 molecular weight species during high performance liquid gel chromatography. Chemical cross-linking of the catalytic subunit with dimethyl suberimidate was performed to determine whether this observation was attributable to a trimeric quaternary structure, an equilibrium between oligomeric forms, or to a non-compact shape. The results of this determination, performed according to the procedure described by Hillel and Wu (1977) are presented in Figure 26. During the time course of chemical cross-linking, the progressive occurrence of dimeric and tetrameric oligomers of M2 is observed in the SDS-polyacrylamide gel. At a cross-linking reaction time of 60 minutes (lane 5), the predominant protein M2 oligomeric forms present are dimer and tetramer. Similar results are also obtained at 240 minutes of sample reaction time. In all cases, no significant amount of trimer was observed. Results from the previous section indicate that native M2 protein exists as a trimeric oligomeric species, on the basis of molecular size. This result can be now accounted for if taken together with the results of the chemical cross-linking experiment. The trimeric behavior of native M2 protein during gel chromatography and the occurrence of dimeric and tetrameric but not trimeric M2 protein species during cross-linking of native catalytic subunit strongly argue that isolated protein M2 exists in a dimer-tetramer equilibrium.

Figure 26. SDS-polyacrylamide gel electrophoresis of calf thymus ribonucleotide reductase catalytic subunit cross-linked with dimethyl suberimidate. Lanes 1 and 7, cross-linked BSA standard. Lane 2, M2 control, 15 ug (specific activity: 3.99 U/mg). Lanes 3-6, M2 cross-linked with dimethyl suberimidate for the indicated time (in minutes). Lanes 3 and 4, 15 ug. Lanes 5 and 6, 30 ug.





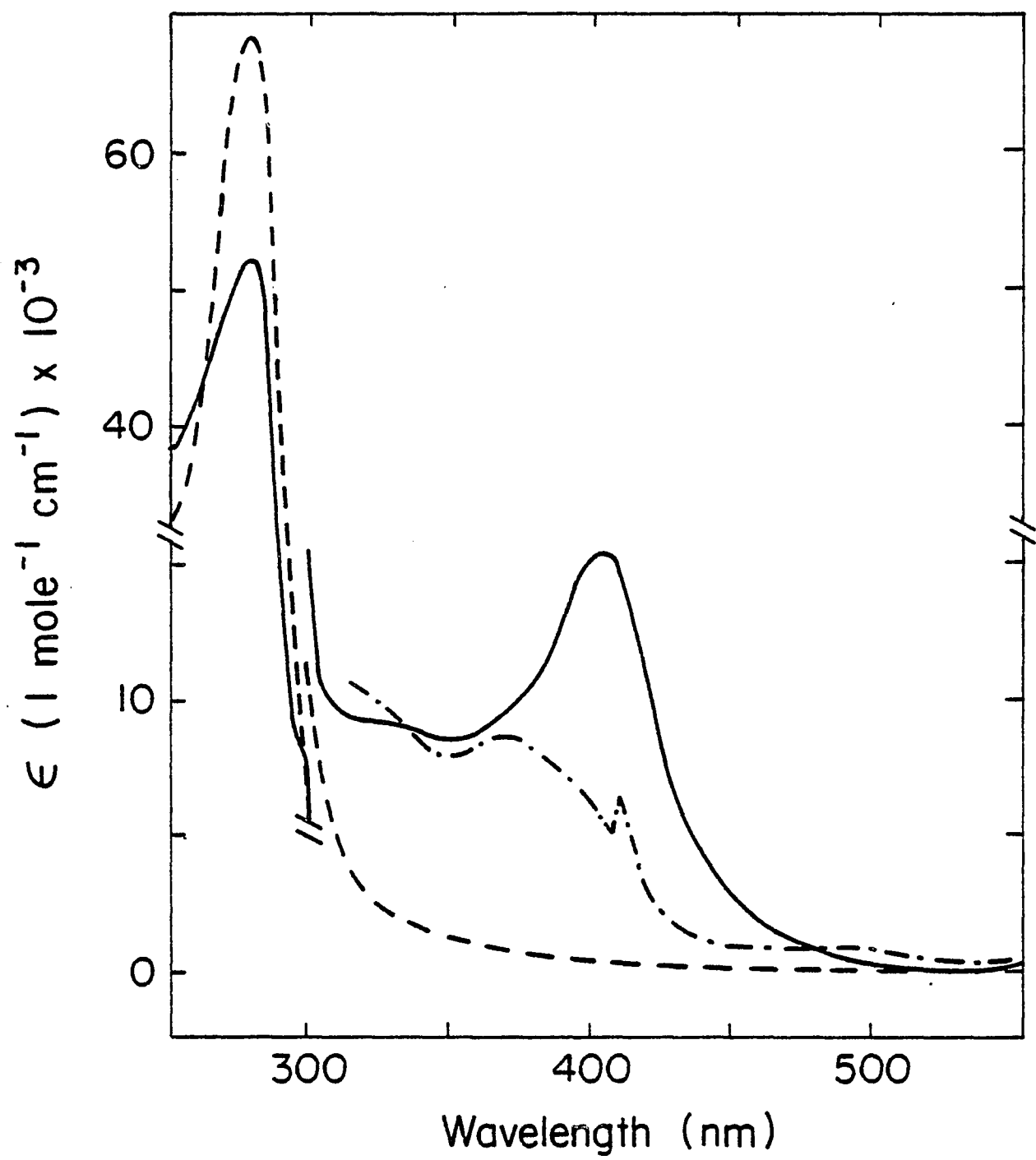
## Spectroscopic Analysis

### Ultraviolet-Visible Spectroscopy

The catalytic subunit of E. coli ribonucleotide reductase exhibits a distinct UV-visible absorption spectrum, with spectral components contributed by both the iron center and the tyrosyl free-radical (Petersson et al., 1980). To determine whether the calf thymus ribonucleotide reductase had any spectral properties in common with the bacterial enzyme, the UV-visible absorption spectra of the M1 and M2 subunits were examined.

The M2 subunit has a characteristic UV-visible absorption spectrum (Figure 27) which includes an intense absorption maximum at 404 nm with a molar extinction coefficient of 15,000 1/M cm. The M1 subunit does not possess this feature but it is seen in the intact enzyme (Figure 30). The B2 spectrum contains a sharp peak at 410 nm, attributed to the tyrosyl free-radical (Petersson et al., 1980). This feature is not evident in the protein M2 spectrum but it well could be hidden under the broad 404 nm transition. Also, addition of hydroxyurea (20 mM final concentration) to the protein M2 sample was without effect, in contrast to the significant spectral changes which occur in E. coli protein B2, due to quenching of the free radical (Atkin et al., 1973). An absorption peak at 370 nm is seen in the B2 subunit but with a lower extinction coefficient than M2. The M2 and B2 subunits each absorb at 325 nm but the extinction coefficients at this wavelength differ considerably as well (it should be noted that molar extinction coefficients are based upon a 78,000 molecular weight dimer of protein B2 while those for calf thymus protein M2 are based

Figure 27. Absorption spectra of the subunits of ribonucleotide reductase: Subunit M2 (—), specific activity: 7.85 U/mg; subunit M1 (-----), specific activity: 4.72 U/mg; E. coli ribonucleotide reductase subunit B2 (· · ·), from Petersson et al., 1980.

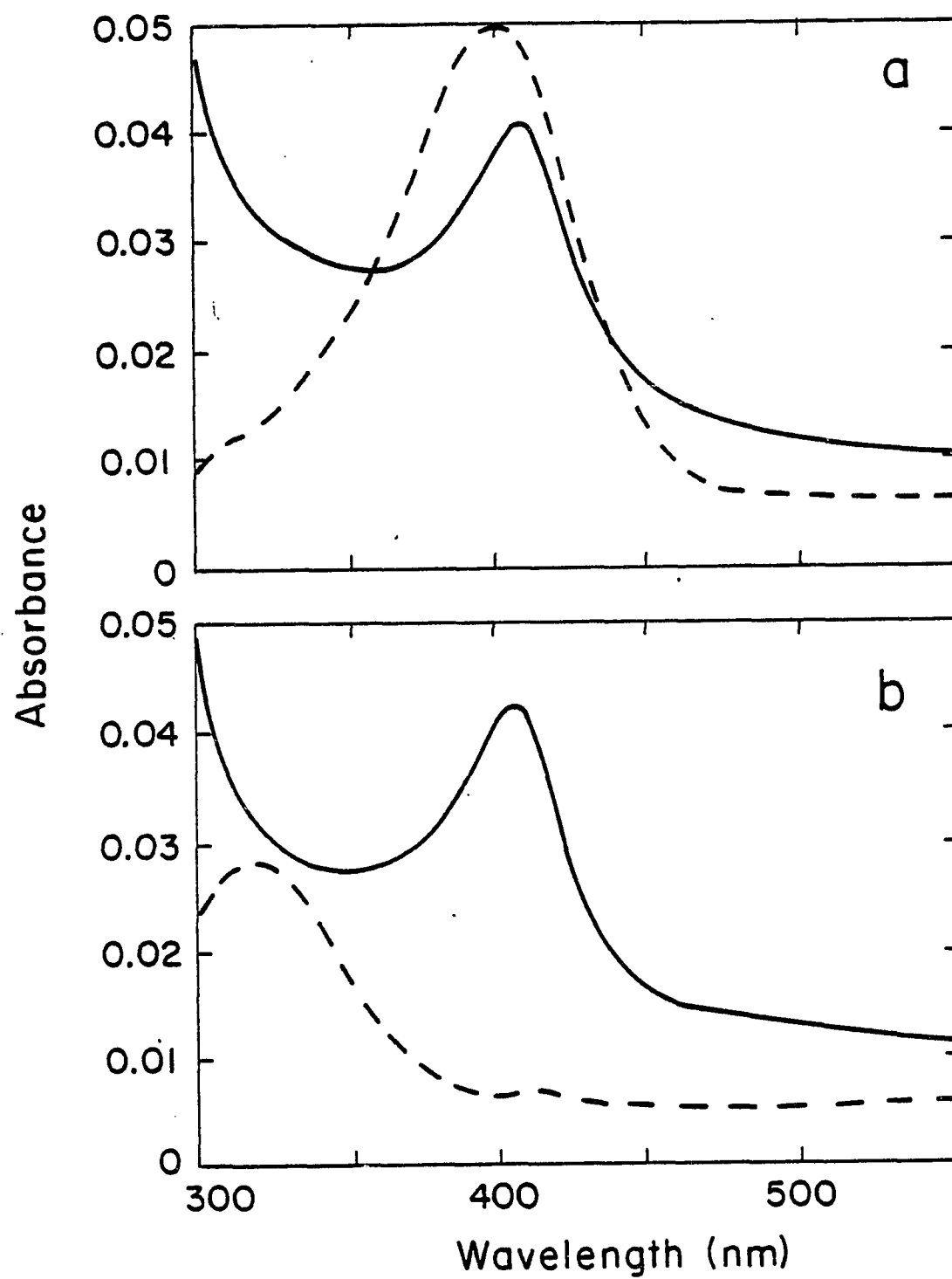


on a 58,000 molecular weight monomer).

The visible chromophore associated with the M2 polypeptide exhibits a spectrum which is analogous to that for the p-nitrophenoxylate anion ( $\epsilon_{398} = 18,300 \text{ l/M cm}$ ; Kezdy and Bender, 1962). P-nitrophenol is utilized as a precursor to the chemical spacer arm linking cyanogen bromide-activated sepharose and dATP in dATP-sepharose (Ward, 1980). Although excess p-nitrophenol is removed from the reaction mixture prior to ligand coupling to the insoluble support matrix (Ward, 1980), the possibility existed that if residual p-nitrophenol were associated with the affinity resin, the compound might specifically interact with protein M2. The resulting p-nitrophenol-M2 protein complex might then give rise to the observed protein M2 visible absorption spectrum under experimental conditions. To test this hypothesis, the effects of pH and a chaotropic salt upon the UV-visible spectral features of protein M2 and the p-nitrophenoxylate anion were evaluated.

Titration of the p-nitrophenoxylate anion ( $\text{pK}_a = 7.04$ ; Kezdy and Bender, 1962) shifts the absorbance maximum from 398 nm to 317 nm. In addition, the extinction coefficient of product p-nitrophenol ( $\epsilon_{317} = 9630 \text{ l/M cm}$ , Kezdy and Bender, 1962) is lower than that of the corresponding anion. This effect, shown in Figure 28, is readily observed when the solvent pH is lowered from 7.6 to 6.0. However, this pH change has no significant effect upon the M2-visible chromophore (Figure 28). Conversely, the presence of 6 M guanidinium hydrochloride causes a significant reduction in the intensity of the 404 nm

Figure 28. The effect of pH upon the visible absorption spectra of ribonucleotide reductase M2 subunit and p-nitrophenoxylate anion: M2 (——) specific activity - 8.46 U/mg; p-nitrophenoxylate anion (-----). A) pH 7.6; solvent: 0.1 M KCl, 50 mM Tris-HCl, 0.1 mM DTE. B) pH 6.0; solvent: 25 mM KCl, 25 uM DTE, 12.5 mM Tris, 0.45 M 2-[N-morpholino]ethanesulfonic acid-HCl.

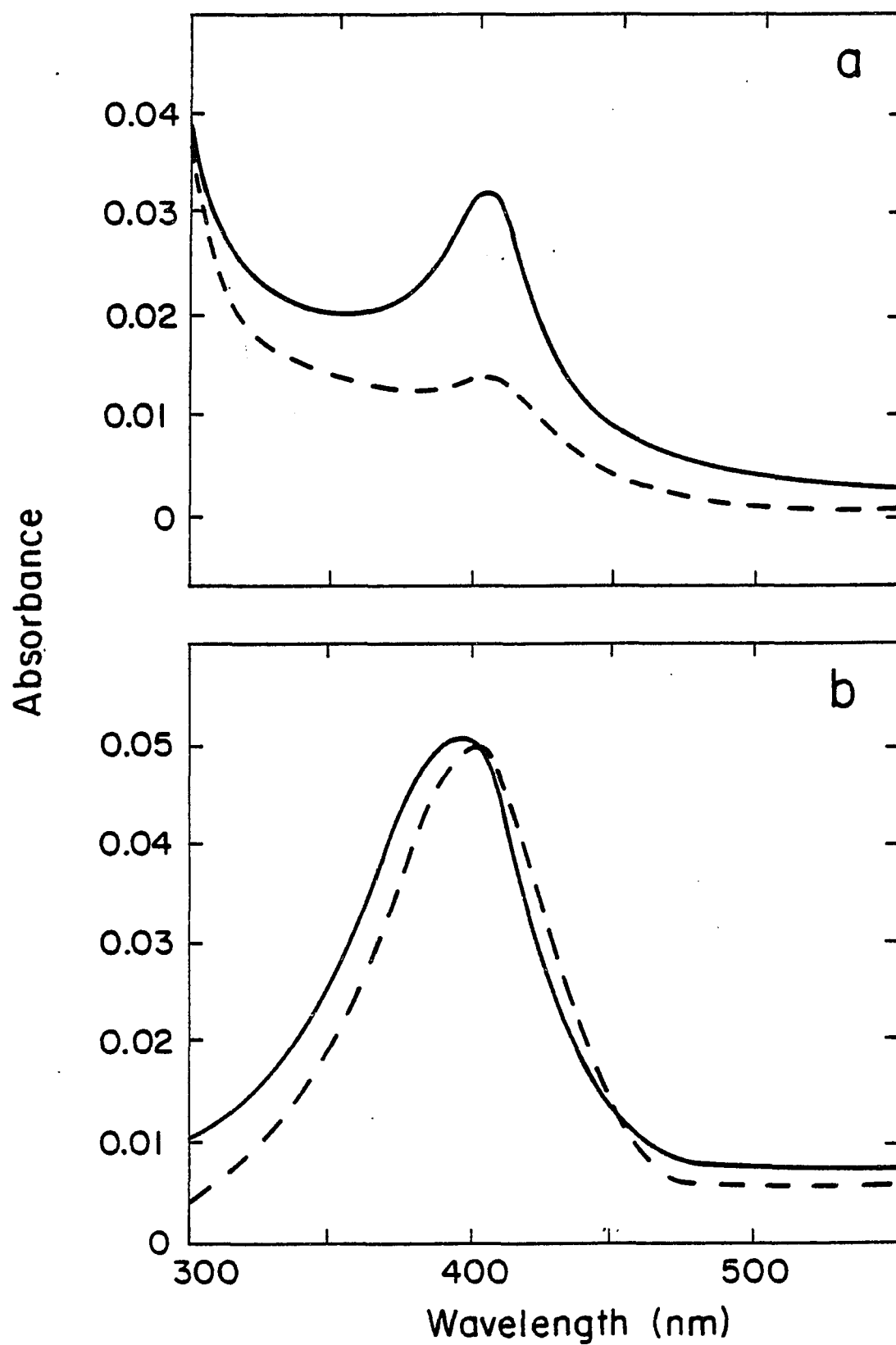


absorption peak associated with the M2 polypeptide (Figure 29), although it does not entirely disappear even under these conditions. The absorption peak of p-nitrophenoxylate is shifted from 398 nm to 404.5 nm when the solvent contains 6 M guanidinium hydrochloride but the absorption intensity remains unchanged. Thus, p-nitrophenoxylate can be ruled out as a possible contributing species to the absorbance of the catalytic subunit in the visible region. Also, the effect of guanidinium hydrochloride on the protein M2 chromophore's absorbance establishes that the chromophore is dependent upon the native protein conformation. The presence of EDTA (0.17 M) or dithionite (2 mM) had no effect upon the spectral properties of the protein M2-associated visible chromophore. Thus, the identity of the moiety giving rise to the visible chromophore in protein M2 is not yet known. However, the dependence of the 404 nm chromophore on the native conformation of protein M2 tends to rule out a stable organic cofactor.

The visible absorption spectrum of the catalytic subunit of E. coli ribonucleotide reductase contains a sharp peak at 410 nm (Figure 27), attributed to the tyrosyl free-radical (Petersson et al., 1980). Since the calf thymus enzyme behaves differently towards inhibitors such as hydroxyurea and 2'-deoxy-2'-azidocytidine diphosphate than does the E. coli enzyme, it has been suggested that the mammalian enzyme may not contain a permanent free radical (Thelander et al., 1980). Attempts to generate a "radical-containing" enzyme species detectable by visible absorption spectroscopy by the addition of various substrates were unsuccessful. The addition of CDP and/or DTE to final concentrations of 0.1 mM and 6.0 mM respectively, had no



Figure 29. The effect of 6 M guanidinium hydrochloride upon the visible absorption spectra of ribonucleotide reductase M2 subunit and p-nitrophenoxylate anion: A) native M2 (188 ug/ml, specific activity: 8.46 U/mg)(————); M2 (188 ug/ml) in 6 M guanidinium hydrochloride (-----). B) p-nitrophenoxylate anion (————); p-nitrophenoxylate anion in 6 M guanidinium hydrochloride (-----). Solvent: 0.1 M KCl, 50 mM Tris-HCl, 0.1 mM DTE, pH 7.6.



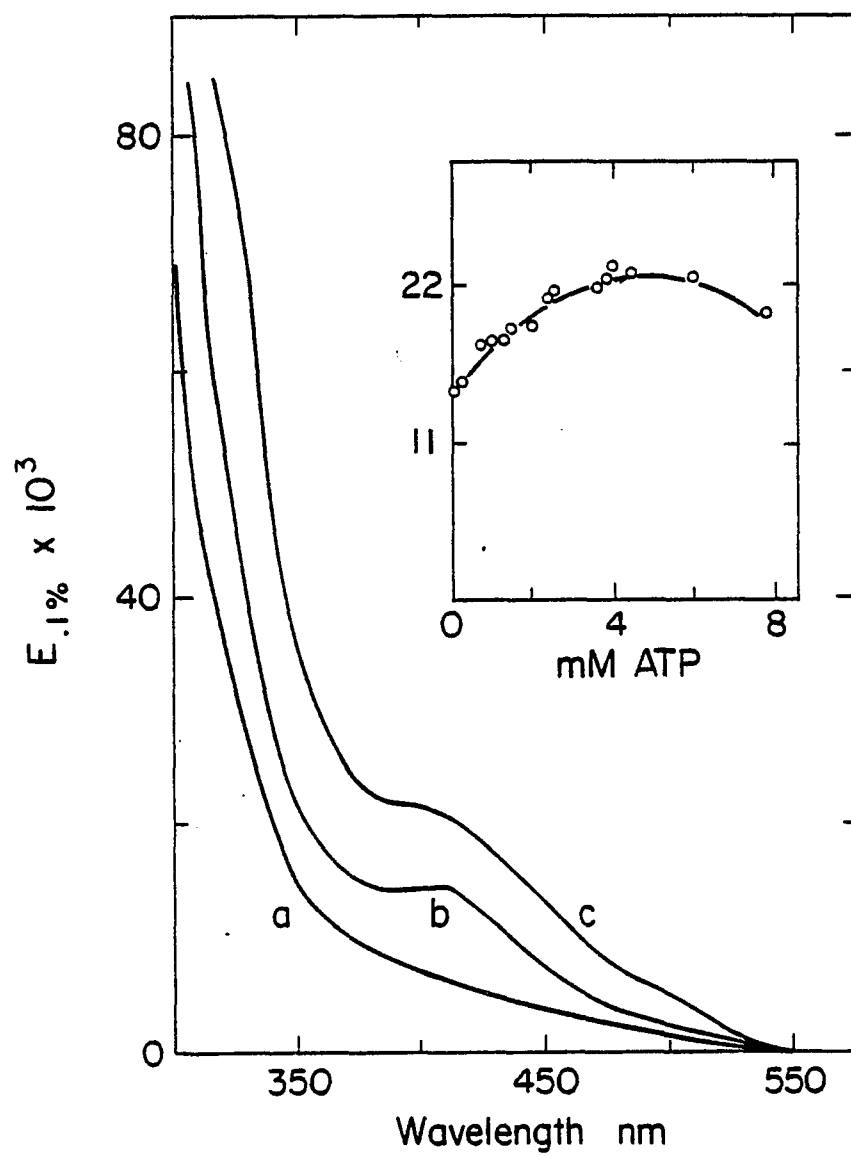
effect upon the visible absorption properties of non-dissociated calf thymus ribonucleotide reductase. However, addition of ATP, a positive allosteric effector, alone or in combination with the other substrates, dramatically increases the absorbance of the intact enzyme in the visible region with a maximal effect at 4 mM ATP (Figure 30). Nevertheless, the resultant spectrum is still unlike that of the tyrosyl radical containing E. coli ribonucleotide reductase (Petersson et al., 1980). ATP in this concentration range alters the protein M1 oligomeric form (Thelander et al., 1980; Klippenstein and Cory, 1978) and presumably shifts the equilibrium toward an active M1M2 protein complex. This spectral change may also reflect a structural change at the chromophore.

#### Electron Paramagnetic Resonance Spectroscopy

The catalytic subunit of E. coli ribonucleotide reductase contains a free radical associated with a tyrosyl residue (Ehrenberg and Reichard, 1972; Sjöberg et al., 1977). A similar radical species has recently been observed in a hydroxyurea-resistant mouse fibroblast 3T6 cell line, which is reported to overproduce the ribonucleotide reductase M2 subunit (Åkerblom et al., 1981; Gräslund et al., 1982). Accordingly, the EPR spectral properties of highly purified non-dissociated ribonucleotide reductase and the enzyme's M2 subunit were examined to determine whether a similar free radical species could be detected in a calf thymus enzyme preparation.

EPR spectroscopy experiments on both the catalytic subunit and the non-dissociated ribonucleotide reductase from calf thymus failed to reveal a free-radical signal analogous to those reported for the

Figure 30. The effect of ATP upon the visible absorption spectrum of non-dissociated ribonucleotide reductase. A) M1 subunit. B) Non-dissociated ribonucleotide reductase, (specific activity: 5.65 U/mg). C) Non-dissociated ribonucleotide reductase, in presence of 6.1 mM ATP. Inset: Absorbance change at 404 nm as a function of ATP concentration. Solvent: 0.1 M KCl, 0.1 mM DTE, 50 mM Tris-HCl pH 7.6.



bacterial enzyme (Ehrenberg and Reichard, 1972) and the mouse fibroblast 3T6 cells (Gräslund et al., 1982). Furthermore the addition of ATP, CDP, and DTE to final concentrations used in the CDP reductase assay, alone and as mixtures, did not result in the generation of a detectable radical species resembling those previously described. However, in one experiment a significant EPR signal attributable to the M2 subunit was observed. As compared to solvent (Figure 31a), a preparation of M2 subunit displayed what appears to be a doublet EPR spectrum centered at  $g = 2.038$  (Figure 31b) which was approximately 14 mT in width from trough to trough. This spectrum is distinct from that reported to occur in ribonucleotide reductase from other sources (Gräslund et al., 1982). Further investigation will be required to determine the catalytic significance and the source of this transition. Also, an attempt to generate a ribonucleotide reductase-associated radical species was made using the method described by Gräslund et al. (1982). Addition of DTE and  $\text{FeCl}_3$  (final concentrations of 10 mM and 10  $\mu\text{M}$ , respectively) to a solution of highly purified non-dissociated calf thymus ribonucleotide reductase (100  $\mu\text{l}$ , 3.9 mg/ml, specific activity: 5.82 U/mg) did not result in the formation of a detectable free-radical signal.

During the course of the EPR spectroscopic experiments, an interaction of iron with CDP reductase assay mix components, specifically ATP and NaF, was found. The EPR spectrum of an  $\text{FeCl}_3$ -ATP-NaF mixture is shown in Figure 32a. Mixture of all three components results in the generation of a broad single absorption centered at  $g = 2.59 \pm .005$ . The microwave power saturation profile is shown in Figure 33.

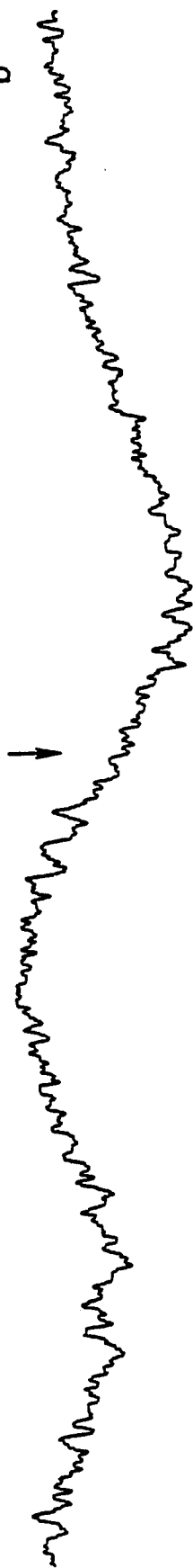
Figure 31. EPR spectra of A) 0.1 M KCl, 0.1 mM DTE, 50 mM Tris-HCl, pH = 7.6 (4 scan signal average); B) calf thymus ribonucleotide reductase M2 subunit (100  $\mu$ l, 0.54 mg/ml, specific activity: 4.32 U/mg, 4 scan signal average). Instrument settings - field set: 330.175 mT, microwave frequency: 9.256 GHz, modulation amplitude: 0.05 mT, receiver gain:  $1.6 \times 10^4$ , microwave power: 0.5 mW, temperature: 77K, scan time: 8 minutes. Arrow indicates position where  $g = 2.0028$ .

a



4 mT

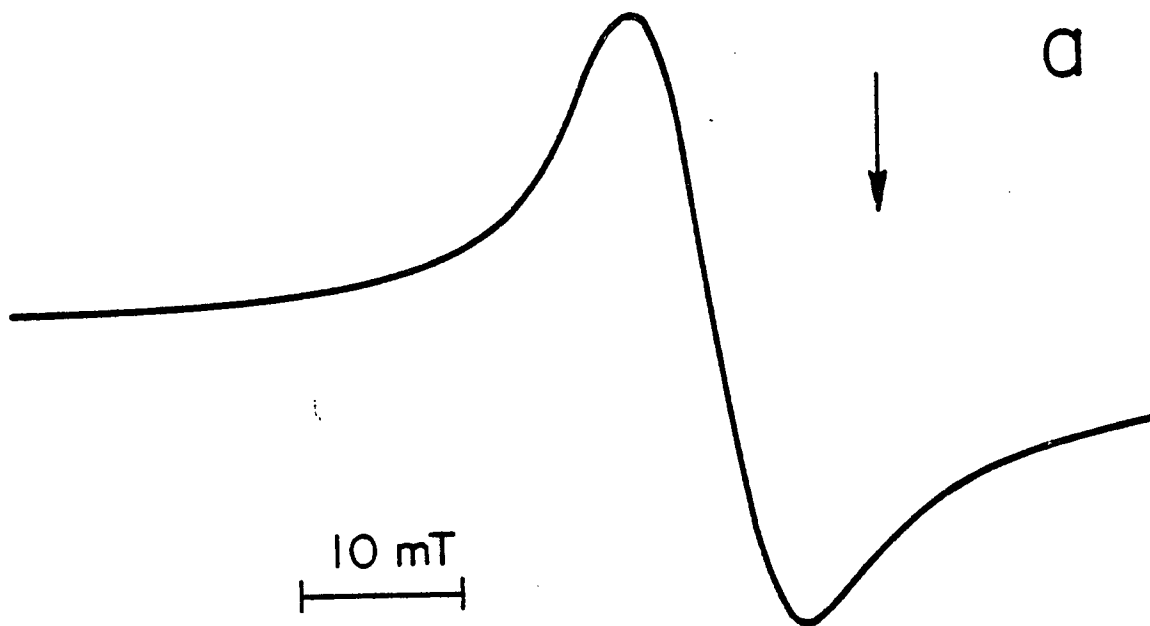
b



H →



Figure 32. EPR spectra of A) 3 mM  $\text{FeCl}_3$ , 2.46 mM ATP, 6.23 mM NaF solution. Instrument settings - field set: 338.65 mT, microwave frequency: 9.388 GHz, modulation amplitude: 0.63 mT, receiver gain:  $2.0 \times 10^3$ , microwave power: 0.5 mW, temperature: 77 K; B) 0.57 mM Fe(II)ascorbate, 1.64 mM ATP, 1.0 mM  $\text{Mg}(\text{CH}_3\text{COO}^-)_2$ , 4.15 mM NaF, 3.0 mM DTE, 0.05 mM CDP solution. Molar ratio of ascorbate to Fe in Fe(II) complex is 20:1 (Atkin et al., 1973). Instrument settings - field set: 330.0 mT, microwave frequency: 9.496 GHz, modulation amplitude: 0.5 mT, receiver gain:  $1.25 \times 10^4$ , microwave power: 1.5 mW, Temperature:  $-185^\circ\text{C}$ .



H →

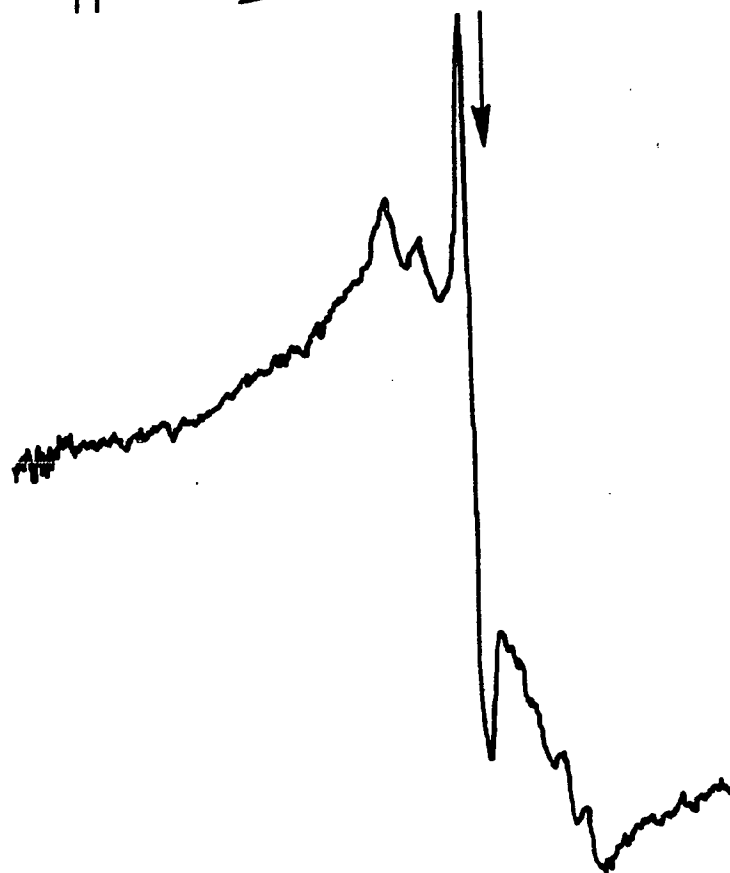
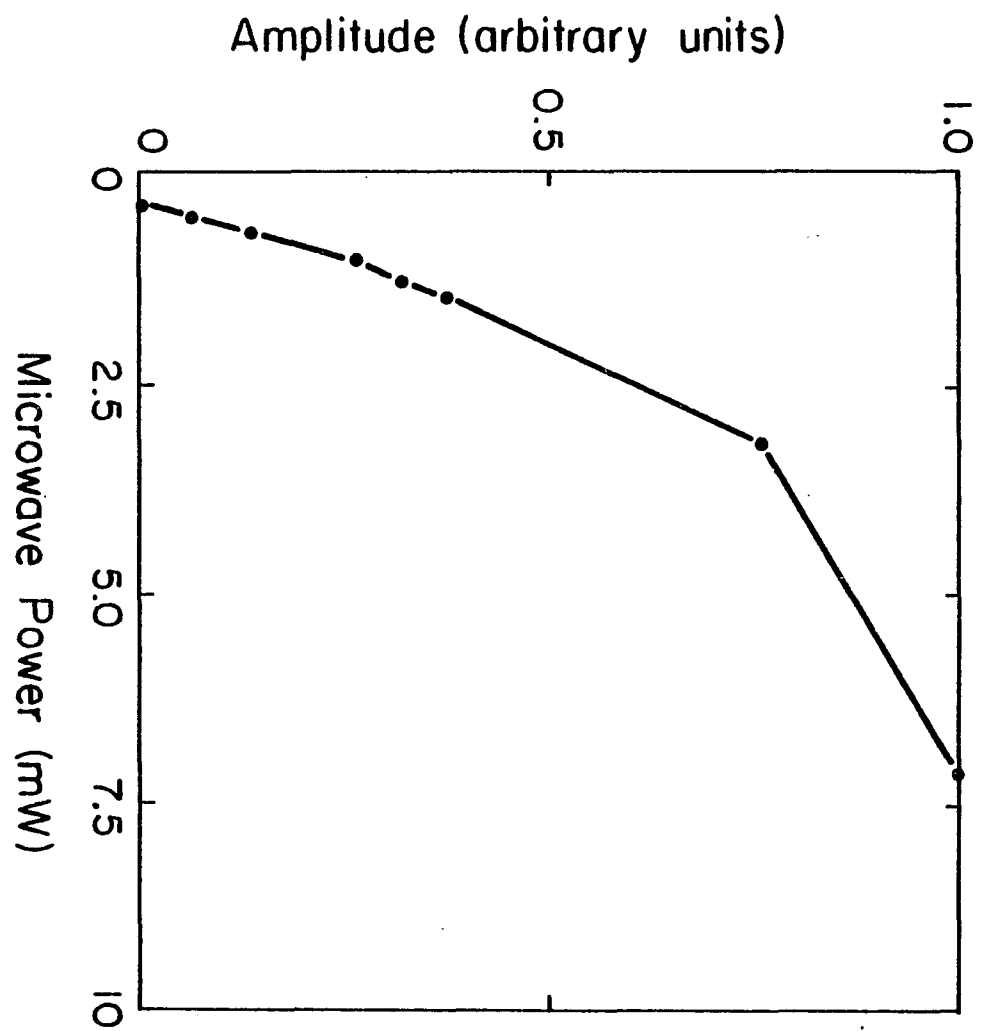


Figure 33. Power saturation profile for  $\text{FeCl}_3$ -ATP-NaF mixture described in Figure 32.



That the curve does not intersect the origin is attributed to a calibration error in the spectrophotometer power setting control. When iron is added to the other two components as Fe(II)ascorbate, a significantly different EPR signal is observed. Figure 32b shows the EPR spectrum of an Fe(II)ascorbate-ATP-NaF mixture. This more complex spectrum consists of sharp transition centered at  $g = 2.010 \pm .005$ , overlying a broader more complex absorbance. In either case, the EPR detectable entity is not believed to be a free radical species since the signal is not observed at 25°C.

#### Effect of the M2 Subunit Upon the Activity of Ribonucleotide Reductase from *E. coli*

Although the regulatory subunits of ribonucleotide reductase from calf thymus (M1) and *E. coli* (B1) have a similar amino acid composition and polypeptide molecular weight, several differences in the physical properties of each are known to occur. These include a reported difference in the number of effector binding sites on each protein (Thelander et al., 1980; Brown and Reichard, 1969b) and an effect of  $Mg^{2+}$  ion upon the sedimentation properties of protein B1 but not protein M1 (Thelander et al., 1980). Also, the activity of the bacterial enzyme is optimized at a higher  $Mg^{2+}$  ion concentration (15 mM; Holmgren et al., 1965) than the calf thymus enzyme (2 mM; Plumer, 1980).

Highly purified non-dissociated calf thymus ribonucleotide reductase and M1 subunit are routinely assayed for protein M1 activity in the presence of an excess of partially purified M2 subunit (Plumer, 1980). However, examination of the activity of mixtures of calf

thymus ribonucleotide reductase protein M2 and the E. coli enzyme reveals that, although the addition of exogenous partially purified protein M2 to a calf thymus ribonucleotide reductase preparation results in an increase in activity (due to excess protein M1 in the preparation), the activity of the E. coli enzyme is inhibited by the presence of an excess of protein M2. The results of this experiment are presented in Table X. Addition of exogenous M2 protein to a calf thymus enzyme preparation doubled the measured enzyme activity. However, at either a 2 mM or 10 mM  $\text{Mg}(\text{CH}_3\text{COO}^-)_2$  concentration, the E. coli enzyme activity is decreased by approximately 70%. When a smaller amount of protein M2 is added to the E. coli ribonucleotide reductase, an increase in activity (~30%) is observed (Figure 34). As the amount of protein M2 added to the E. coli ribonucleotide reductase assay is increased, enzyme inhibition becomes apparent. This effect may be related to the reported inhibition of ribonucleotide reductase by an excess of protein M2 (Thelander et al., 1980). Alternatively, it may result from the presence of another component, as yet unidentified, present in the partially purified M2 subunit preparation.

TABLE X.

Comparative Effect of M2 on the Activity of Ribonucleotide Reductase  
from Calf Thymus and E. coli, at two  $Mg^{2+}$  Ion Concentrations

Enzyme Source	Units/Assay X $10^2$			
	2mM <sup>*</sup>		10mM <sup>*</sup>	
Calf thymus	1.2	(100)	0	--
	2.4 <sup>+</sup>	(200)	0	--
<u>E. coli</u>	6.6	(100)	31.8	(100)
	2.0 <sup>+</sup>	( 31)	8.4 <sup>+</sup>	( 26)

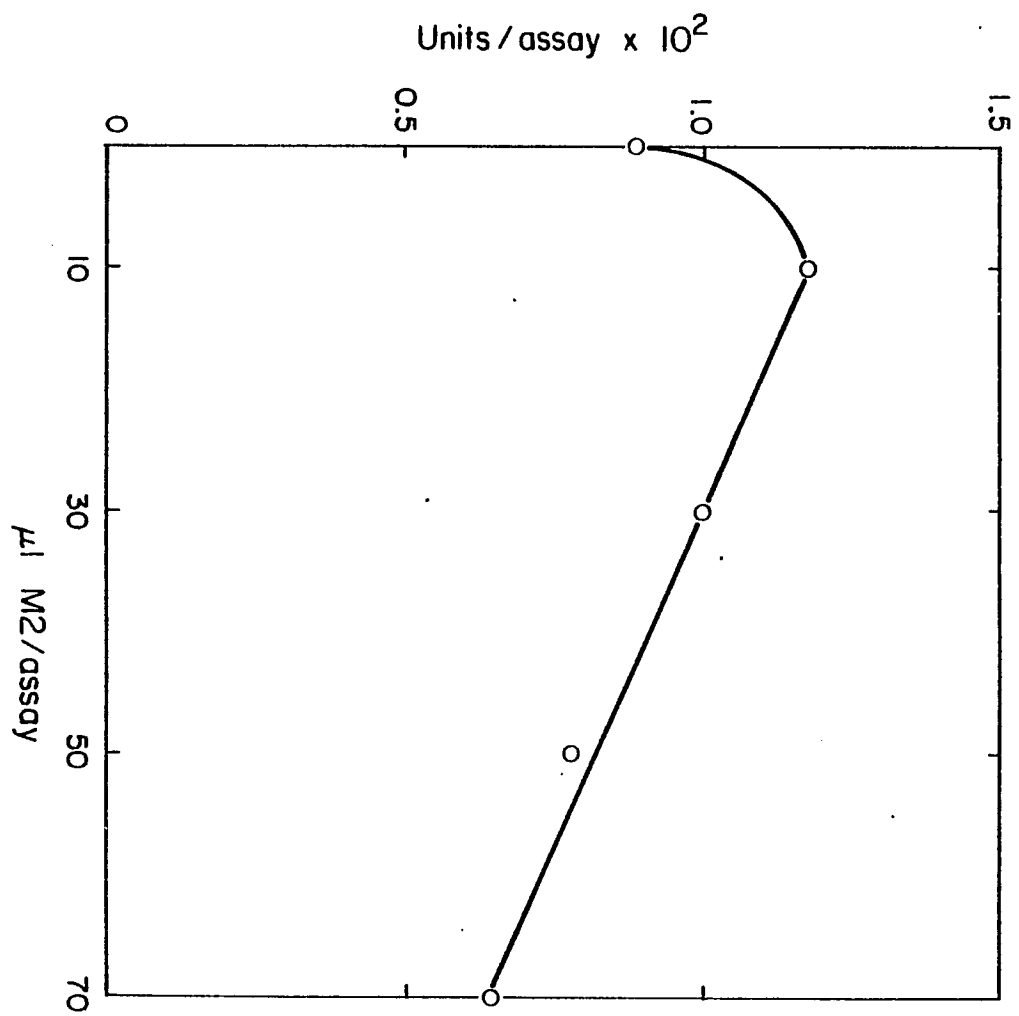
\*concentration of  $Mg(CH_3COO^-)_2$  in assay.

<sup>+</sup>assayed in the presence of M2 (0.012 units)  
prepared according to Plumer (1980).

numbers in parenthesis indicate activity as percent of original.

Figure 34. Effect of protein M2 upon the activity of E. coli ribonucleotide reductase. M2 activity: 0.10 U/ml. The concentration of  $\text{Mg}(\text{CH}_3\text{COO}^-)_2$  in the assay was 10 mM.f





## VI. DISCUSSION

The iron-requiring ribonucleotide reductases from E. coli (Brown et al., 1967) and mammalian sources including rabbit bone marrow (Hopper, 1972), calf thymus (Thelander et al.), 1980, Ehrlich tumor cells (Cory et al., 1980), and regenerating rat liver (Youdale and MacManus, 1981) are comprised of two non-identical subunits. The two subunits are enzymatically inactive alone but yield active enzyme when combined. The purification strategy for several ribonucleotide reductases has been either to separately purify the complementary subunits, e.g., E. coli (Brown et al., 1969b) or to design a protocol which minimizes separation of the subunits, e.g., calf thymus (Engström et al., 1979). The protein designations M1 or B1 and M2 or B2 have been introduced to differentiate between the effector binding (regulatory) and non-heme iron containing (catalytic) ribonucleotide reductase subunits, respectively (Brown et al., 1967; Plumer, 1980; Thelander et al., 1980). The prefix M or B refers to the enzyme source, e.g., mammalian or bacterial. This convention, which is convenient for comparative purposes, has been utilized throughout this study.

Engström et al. (1979) have reported the purification of non-dissociated calf thymus ribonucleotide reductase. The enzyme preparation was characterized by an apparent non-stoichiometry of the catalytic (M2) subunit with respect to the regulatory (M1) subunit. SDS-polyacrylamide gel electrophoresis indicated that protein M2 was a minor component in the preparation. Subsequently, homogeneous protein M1 was obtained by separation of the M1 and M2 subunits of partially

purified calf thymus enzyme on a column of Blue Sepharose, followed by chromatography of the M1 preparation on dATP sepharose (Thelander et al., 1980). The M2 subunit was not obtained in homogenous form.

Plumer (1980) has described an alternate method of preparing highly purified calf thymus ribonucleotide reductase M1 subunit. This procedure is unique in that it achieves the chromatographic separation of the complementary subunits of a crude enzyme preparation on a DEAE-cellulose column in the presence of ATP and  $Mg^{2+}$ , prior to sequential affinity chromatography of the protein M1-containing fraction on ATP-agarose in the presence and absence of  $Mg^{2+}$ . Further purification of the M2 subunit was not performed.

Although established methods for the purification of the regulatory subunit of ribonucleotide reductase were available (Plumer, 1980; Thelander et al., 1980), a method for the preparation of homogenous catalytic subunit had not been reported. Accordingly, the initial focus of this research was on the preparation of highly purified M2. However, the objective of this research was to investigate the physicochemical characteristics of the complementary subunits of ribonucleotide reductase from calf thymus.

Attempts to further purify protein M2, starting with crude preparations obtained by the method of Plumer (1980), by chromatography on DEAE-cellulose were without success since the subunit is not highly resolved during gradient-elution of the column. In contrast, a purification of the M2 subunit was obtained by preparative isoelectric

focusing and sucrose gradient centrifugation. A fraction of  $pH_I = 5.5$  exhibited enzyme activity when combined with protein M1. The preparation sedimented as a major protein peak of 6.4 s during sucrose gradient centrifugation. Analysis of the 6.4 s fractions on gradient gel electrophoresis revealed two components of molecular weight ( $M_r$ ) = 107,000 and 160,000. However, the final protein yield was very low (< 75 ug) and in addition, the protein M2 activity was unstable to gradient centrifugation at this level of purity. Therefore, a method for obtaining the M2 protein from purified non-dissociated ribonucleotide reductase was sought.

#### Purification of Ribonucleotide Reductase from Calf Thymus

The purification procedure for intact ribonucleotide reductase from calf thymus was patterned after that of Engström et al. (1979) with modification of the DEAE-cellulose column step as described by Plumer (1980). Furthermore, the protocol was simplified by deletion of the hydroxylapatite column procedure and replacement of the gel chromatography desalting step with dialysis. Comparison of the results for the purification of calf thymus ribonucleotide reductase using the simplified procedure described above and the original method of Engström et al. (1979) indicates that both protocols yield products of similar quality and amount. The yield of total enzyme units from the ammonium sulfate precipitation step, approximately 50% as compared to the tissue homogenate, was significantly less than the near quantitative recovery reported previously (Engström et al., 1979). Also, after normalization of the specific activity data to compensate for differences in the CDP concentration of the ribonucleotide reductase

assay (Plumer, 1980) as compared to that of Engström et al. (1979), the specific activity of enzyme prepared by the modified procedure described above is approximately one third of that reported for the original procedure. For a highly purified enzyme preparation (approximately 3000 fold), this difference is probably not significant. Also, SDS-polyacrylamide gel electrophoresis of the highly purified ribonucleotide reductase indicates that the M2 subunit occurs in substoichiometric quantities in the intact enzyme preparation, a finding consistent with previous results (Engström et al., 1979).

#### Purification of the Complementary Subunits of Ribonucleotide Reductase

ATP has been shown to induce changes in the sedimentation behavior of ribonucleotide reductase from calf thymus (Eriksson et al., 1977), human cells (Chang and Cheng, 1979), and Ehrlich tumor cells (Klippenstein and Cory, 1978). Plumer (1980) reported an activation of the partially purified calf thymus enzyme by 4 mM ATP and inhibition of the enzyme activity at higher nucleotide concentrations. These observations were proposed to result from an ATP-induced enzyme dissociation (Plumer, 1980). A modified method consisting of ion-exchange chromatography in the presence of ATP was then developed to separate the complementary subunits of crude calf thymus ribonucleotide reductase. This method takes advantage of the changes induced in the oligomeric properties and charge of the enzyme under these conditions (Plumer, 1980). The binding of ATP to the M1 subunit (Thelander et al., 1980) of ribonucleotide reductase in the presence of 4 mM ATP and 4 mM  $\text{Mg}(\text{CH}_3\text{COO}^-)_2$  allowed for the nearly complete separation of the M2 subunit from the M1 subunit activity. This is

due to the fact that the M1 subunit is more strongly absorbed onto DEAE-cellulose under these conditions (Plumer, 1980). This procedure was thus adapted as a means of dissociating the complementary subunits of the highly purified ribonucleotide reductase, prepared as described.

Application of this "splitting column" methodology to the ribonucleotide reductase obtained from dATP-Sepharose chromatography results in the preparation of homogenous protein M2 as well as protein M1. This is the first report of a method by which the catalytic (M2) subunit can be obtained in highly purified form. Each polypeptide runs as a single band in SDS-polyacrylamide gel electrophoresis with molecular weights ( $M_r$ ) of 84,000 for M1 and 58,000 for M2. The polypeptide molecular weight of M1 is in good agreement with previous work (Plumer, 1980; Thelander et al., 1980). The specific activity of the M1 protein is about 25% of that previously obtained by Thelander et al. (1980) but nearly twice that obtained by the procedure of Plumer (1980). However, the specific activity of the M2 subunit, prepared as described above is approximately 15 times greater than that reported for a partially purified protein M2 fraction (Thelander et al., 1980) and is the highest reported thus far for the catalytic subunit of a eukaryotic ribonucleotide reductase.

There is enzyme activity in the isolated protein M1 preparation. This is attributed to incomplete dissociation of the ribonucleotide reductase. Since the presence of protein M2 was not detected in this fraction on SDS-polyacrylamide gels, the amount of protein M2

contamination in this pool is thought to be insignificant in relation to protein M1. However, activity measurements do show the presence of measurable activity. Thus the presence of intact enzyme activity in this preparation indicates the ability of the ribonucleotide reductase to exhibit significant activity under conditions where the subunit ratio is far from unity.

#### Complementary Subunit Physical Composition

Comparison of the amino acid composition data for the subunits of ribonucleotide reductase from the calf thymus and E. coli indicates that significant compositional homology (Marchalonis and Weltman, 1971; Cornish-Bowden, 1981) exists between the regulatory, but not the catalytic, subunits of the enzyme. Although the M1 and B1 proteins are related by similar function, size, and amino acid composition, several distinct differences between these two polypeptides have been described. These dissimilarities include a reported difference in the number of allosteric effector binding sites per calf thymus protein M1 dimer as compared to E. coli protein B1 dimer, and an effect of  $Mg^{2+}$  upon the sedimentation properties of the bacterial, but not the mammalian, enzyme regulatory subunit (Thelander, 1973; Thelander et al., 1980).

Comparative analytical peptide mapping of the regulatory subunits of ribonucleotide reductase from calf thymus and E. coli was performed to investigate the possibility that these two polypeptides might be structurally related. The results clearly indicate distinct differences in the primary structure of each protein. This finding under-

scores the necessity for additional structural information, to be used in combination with compositional data, when evaluating the possibility for structural homology between polypeptides of different origin. However, the possibility remains that certain functional domains may have been conserved and are common to both polypeptides. Examination of the cross-reactivity of antibodies, directed against the regulatory subunit of the E. coli and calf thymus enzymes, toward the respective antigens might be a useful means to test this hypothesis. Additional insight with regard to this question will obviously be gained by a comparison of the sequence data for these proteins when the information becomes available.

#### Ribonucleotide Reductase Complementary Subunit Solution Hydrodynamics

A polypeptide molecular weight of protein M1 from SDS-polyacrylamide gel electrophoresis of 84,000 and a slightly lower value from analytical gel chromatography were obtained. Since the native and denatured proteins have similar molecular weights, this indicates that, in the absence of allosteric effectors, the native form occurs primarily as a monomer. These results are in agreement with previous work (Thelander et al., 1980).

A polypeptide molecular weight of 58,000 has been determined for the M2 protein. Analytical gel chromatography of native protein M2 gave a Stoke's radius of 53 A. This is in reasonable agreement with the 48 A value attributed to a 110,000 molecular weight dimer (Thelander et al., 1980). However, when this result was computed with respect to molecular weight, a value (185,000) more consistent with a



trimeric oligomeric form was obtained. Further examination of the oligomeric properties of native M2 protein, by cross-linking with dimethyl suberimidate, revealed that the purified M2 subunit exists in a dimer-tetramer equilibrium.

Plumer (1980) has reported the activity of protein M2 in crude preparation to be associated with molecular weight species of 87,000 and 100,000 during gel chromatography on Biogel A 1.5 M and Sephacryl S-300, respectively. These results are consistent with the observation that protein M2 behaves as a dimeric species (Thelander et al., 1980) or possibly exists in a monomer-dimer equilibrium. However, when the data were computed with respect to Stoke's radius, Plumer (1980) obtained values of 35.9 A and 35.8 A for the M2 protein. These are in contrast to the 53 A value reported here for purified protein M2, attributed to an M2 subunit dimer-tetramer equilibrium and the 48 A value (Thelander et al., 1980), attributed to M2 dimer. When compared to similar data for BSA ( $M_r = 67,000$ ;  $R_s = 35.5$  A), the result of Plumer (1980) suggests that the M2 protein ( $M_r = 58,000$ ) is monomeric, or in monomer-dimer equilibrium. A crude preparation of less concentrated M2 protein was used in these determinations. Thus, the oligomeric properties of the M2 polypeptide may be a reflection of the state of protein M2 purity or concentration, relative to other proteins.

The sedimentation behavior of protein M1 is altered by the presence of allosteric effectors, whereas that of protein M2 is not (Thelander et al., 1980). The activity of purified calf thymus ribo-

nucleotide reductase, which contains a substoichiometric amount of M2 subunit, was associated with a 10 s species during analytical glycerol gradient centrifugation, in the presence of dTTP (Engström et al., 1979). However, the activity profile did not correspond to the protein profile (~9 s peak) but correlated with the result expected for a species heavier than the major protein component, M1. This result was interpreted as indicating the occurrence of an active (M1-M2) complex, sedimenting more rapidly than the predominant M1 protein species formed in the presence of dTTP. Highly purified protein M1 was subsequently shown to behave as a dimeric species (8.8 s) in the presence of this allosteric effector (Thelander et al., 1980).

The observations that purified protein M2 occurs as a dimeric species, possibly in equilibrium with a higher oligomeric form, in conjunction with similar results obtained by Thelander et al. (1980) and the demonstration that active ribonucleotide reductase is heavier than dimeric protein M1 (Engström et al., 1979) can be used to predict a model for the quaternary structure of calf thymus ribonucleotide reductase. The combined data strongly suggest that the active enzyme is formed by the association of dimeric species of proteins M1 and M2, respectively, to form a tetrameric complex. If so, then the quaternary structure of the calf thymus enzyme is homologous to the structure of the E. coli ribonucleotide reductase, which has previously been shown to contain dimeric B1 subunit in association with dimeric B2 subunit (Thelander and Reichard, 1979). The behavior of highly purified M2 protein as a species in equilibrium between dimer and

tetramer may in fact result from a self-association of dimeric M2 protein in the absence of complementary subunit or even other proteins. Thelander et al. (1980) have reported a decrease in enzyme activity when an excess of protein M2 is added to the M1 subunit. A possible explanation of these results may be that when protein M2 is present in a more concentrated solution, the equilibrium of oligomeric forms may shift toward the tetrameric M2 species, presumably inactive.

An increase in the extinction coefficient of the chromophore absorbing at 404 nm in intact ribonucleotide reductase is observed when ATP is added to the preparation. This effect is maximized at 4 mM ATP concentration. The CDP reductase activity profile of the calf thymus enzyme is also maximized at this level of allosteric effector (Plumer, 1980). Therefore, under these conditions, the chromophore may function as a reporter group indicating the formation of an active protein M1-M2 complex, postulated here to be  $(M1)_2-(M2)_2$ . Complex formation may also induce a structural change at the chromophore resulting in an increased extinction coefficient. The M1, but not the M2, subunit of calf thymus ribonucleotide reductase binds nucleotides (Thelander et al., 1980). Therefore, it would have to be that ATP binding to the M1 subunit influences the chromophore on the complementary subunit. This inter-subunit effect might be transmitted by and result in a conformational change of the respective complementary subunits in the active enzyme complex.

The model described above for the oligomeric structure of active calf thymus ribonucleotide reductase would also predict an enzyme

molecular weight of 284,000, based upon the polypeptide molecular weights of M1 (84,000) and M2 (58,000). Youdale et al. (1982) have reported a molecular weight of 280,000 for the intact enzyme from regenerating rat liver but comprised of subunits with molecular weights of 45,000 for protein M1 and 120,000 for protein M2, respectively. More recently, Cory and Fleischer (1982) have reported a molecular weight of 304,000 for CDP reductase comprised of subunits with molecular weight of 127,000 for M1 and 77,000 for protein M2, as determined by sedimentation equilibrium of partially purified Ehrlich tumor cell ribonucleotide reductase. Also, an ADP reductase of 254,000 molecular weight, from the same tissue source, comprised of subunits with molecular weight of 95,000 for protein M1 and 85,000 for protein M2, was described. These authors have proposed that intact mammalian ribonucleotide reductase is made up of an M2 protein component(s) which is common for all substrates and an M1 protein component(s) which are specific for each substrate. However, Chang and Cheng (1979) and Eriksson et al. (1979) have reported that only one protein M1 species catalyzes the reduction of all four ribonucleoside diphosphates. These protein M1 preparations may be resolvable into more substrate specific components (Cory and Fleischer, 1982). Therefore, in addition to differences in the reported molecular weight of the mammalian ribonucleotide reductases and their component subunits, agreement on the number of species of protein M1 has not, as yet, been achieved.

#### The Role of Iron in the Ribonucleotide Reductase Mechanism

In contrast to results reported by Engström et al. (1979), the activity of highly purified ribonucleotide reductase, prepared as

described above, was stimulated nearly two fold by the addition of exogenous iron, an effect reported for the enzyme from other mammalian tissues (Hopper, 1972, 1977; Moore, 1977). A similar stimulation of activity by added cofactor was reported for homogenous protein M1 assayed in the presence of partially purified protein M2 (Thelander et al., 1980). The requirement of protein M2-associated iron for calf thymus ribonucleotide reductase activity has been clearly established (Plumer, 1980). In this study, the differential effect of a metal chelating agent upon the complementary subunits was examined. Treatment of partially purified protein M2, but not protein M1, with EDTA results in complete enzymatic inactivation when the complementary subunits was re-mixed for assay. Thus, these results demonstrated that the ribonucleotide reductase iron cofactor was specifically protein M2-associated (Plumer, 1980). EDTA may inhibit enzyme activity by forming a complex with the protein M2-associated iron in situ or by removing the metal cofactor. Re-activation of EDTA-treated calf thymus ribonucleotide reductase with  $^{59}\text{Fe(II)ascorbate}$  has been described (Engström et al., 1979). A peak of radioactivity cosediments with the protein during gradient centrifugation. Thus, the chelating agent may remove at least some iron from an iron binding domain of ribonucleotide reductase.

Atomic absorption analysis indicates that highly purified calf thymus ribonucleotide reductase M2 subunit contains 3 g-atoms Fe per mole of protein M2 monomer. This result is significantly different from the reported 1 g-atom Fe per mole of E. coli protein B2 monomer (Brown et al., 1969a). Since the addition of exogenous iron to the

enzyme assay stimulates calf thymus ribonucleotide reductase activity, protein M2-associated iron may be either functional or non-specifically bound. That the degree of stimulation is typically nearly two fold may indicate that approximately 50% of the catalytically active M2 protein contains a full complement of iron. However, it is not clear whether exogenous iron stimulates non-EDTA treated enzyme activity by interacting with iron already associated with the M2 subunit, by occupying vacant metal binding domains, or by forming a complex with substrate(s).

Since the calf thymus enzyme behaves differently toward inhibitors such as hydroxyurea and 2'-deoxy-2'-azidocytidine diphosphate than the E. coli ribonucleotide reductase, it has been suggested that the calf thymus enzyme may not contain a permanent free radical (Thelander et al., 1980). Recent EPR studies involving hydroxyurea-resistant mouse fibroblast 3T6 cells (Åkerblom et al., 1981) and pseudorabies-infected mouse fibroblast L cells (Lankinen et al., 1982) have demonstrated the occurrence of a characteristic EPR signal resembling that of the B2 subunit of E. coli ribonucleotide reductase. In the case of the hydroxyurea resistant cell line, isotopic substitution experiments and EPR spectral analysis demonstrated that a tyrosyl free-radical is localized in the M2 subunit (Gräslund et al., 1982).

Although the sensitivity to hydroxyurea was the same for both the mammalian and bacterial enzymes, ribonucleotide reductase from both calf thymus and mouse fibroblast cells was apparently 75 times more

sensitive to 2,3,4-trihydroxybenzohydroxamic acid than the E. coli enzyme (Kjoller-Larsen et al., 1982). Also, both inhibitors act reversibly with the mammalian but not the bacterial enzyme. The differences in reactivity of the polyhydroxybenzohydroxamic acid, which like hydroxyurea acts as a radical scavenger, towards the mammalian and E. coli ribonucleotide reductase is significant. Kjoller-Larsen et al. (1982) have suggested that this may reflect different topologies of the active sites with the site in the mammalian enzyme being more exposed. Such a difference might explain the non-productive interaction (inhibition) of the B1 and M2 subunits, in mixing assays of E. coli ribonucleotide reductase and crude protein M2. The formation of a protein B1-M2 complex may be catalytically inactive, due to a mismatch in structural elements from each complementary subunit contributing to the enzyme active center.

The tyrosyl free-radicals of both E. coli and T4-induced E. coli ribonucleotide reductase are stabilized by the presence of a binuclear Fe(III) center, antiferromagnetically coupled through a  $\mu$ -oxo bridge (Petersson et al., 1980; Sjöberg et al., 1982). The binuclear iron center has also been postulated to also occur in protein M2 from mammalian sources (Gräslund et al., 1982). However, the results reported here have revealed several distinct physicochemical differences between protein M2 from calf thymus and protein B2 from E. coli. Specifically, dissimilarities in the polypeptide size, amino acid composition, UV-visible spectral absorption properties, and iron content of the respective proteins M2 and B2 are indicated.

Gräslund et al. (1982) have recently described an interesting difference in the microwave saturation properties of the tyrosyl free-radical associated with the catalytic subunit of ribonucleotide reductase from hydroxyurea-resistant mouse fibroblast 3T6 cells and from E. coli. During an EPR experiment, a microwave power-saturation effect is identified as a decrease in corrected EPR signal amplitude as the spectrophotometer microwave power is increased. Power saturation is due to an insufficient relaxation of resonant unpaired electrons equally populating two different spin states to a thermal equilibrium where the two spin states are populated slightly differently as defined by Boltzman's law. The relaxation process is normally facilitated in the presence of a paramagnetic species near the free radical under study or by an increase in temperature. Accordingly, the observation that the protein M2, but not protein B2, associated tyrosyl free-radical signal was hardly affected at all by microwave saturation was noteworthy (Gräslund et al., 1982). Since these experiments were performed at a low temperature (77 K), the results suggest that the unusual free-radical non-power saturation for the mammalian enzyme is due to radical interaction with another paramagnetic species.

Examination of the magnetic susceptibility properties of protein B2 has demonstrated that the binuclear iron center of the subunit does exhibit some temperature-dependent paramagnetism (Petersson et al., 1980). However, at 77 K and lower the protein B2 sample magnetic susceptibility did not deviate significantly from Curie law behavior, which predicts a linear relationship between magnetic susceptibility



and the inverse of the temperature. These results are consistent with protein B2 containing an antiferromagnetically-coupled Fe(III) pair which at these temperatures is only significantly populated in the diamagnetic ground state. The magnetic susceptibility properties of protein B2 are therefore only attributable to the organic free radical and other paramagnetic ions associated with the protein. If the binuclear iron center also occurs in protein M2 and exhibits similar properties, then this diamagnetic moiety (at  $T < 77$  K) would not affect the relaxation rate of the protein M2-associated tyrosyl free-radical. Thus, the power non-saturation behavior of the protein M2 radical species would result from tyrosyl free-radical interaction with other paramagnetic ions associated with the subunit.

Atomic absorption analysis of homogenous protein M2 from calf thymus indicates the presence of 6 g-atoms Fe per mole of protein M2 dimer. If two of these iron atoms are associated with the putative antiferromagnetically coupled binuclear Fe(III) center of the dimeric M2 subunit, then the unaccounted for iron atoms may be located in another type of iron binding domain(s). Furthermore, if the domains contained the paramagnetic  $\text{Fe}^{3+}$  species, then the metal might interact with the protein M2 tyrosyl free-radical. This interaction would explain the facilitated relaxation and concomitant non-power-saturation behavior of the protein M2 radical at 77 K (Gräslund et al., 1982).

Comparison of the iron activation effect, the iron content of, and the spectral properties of calf thymus protein M2 with beef heart

aconitase, another non-heme iron-containing protein, suggests the occurrence and identity of a possible paramagnetic iron center of the mammalian ribonucleotide reductase. The role of non-heme iron in the activation and catalytic mechanism of beef heart aconitase has been the subject of both speculation and debate (Villafranca and Milvdan, 1971; Glusker, 1972). However, Kurtz et al. (1979) have recently demonstrated that aconitase-associated iron occurs as a ferredoxin-type binuclear iron cluster. The single  $[\text{Fe}_2\text{S}_2(\text{RS})_4]^{2-,3-}$  cluster per aconitase monomer is analogous to those previously reported to occur in the spinach chloroplast ferredoxin (Orme-Johnson and Orme-Johnson, 1978). Aconitase preparations containing the iron-sulfur cluster in the oxidized (2-) form are enzymatically inactive and exhibit a characteristic EPR signal centered at  $g = 2.01$ , similar to that observed in the "High Potential" ferredoxins from bacteria. Reduction of the ferredoxin-type Fe-S cluster yields active aconitase but abolishes the EPR signal. Both cysteine and ascorbate are efficient reducing agents, but only when ferrous iron is present (Ruzicka and Beinert, 1978). It is not clear whether any of the iron is incorporated into the aconitase active center or whether exogenous iron facilitates efficient Fe-S cluster reduction.

The electronic spectrum of aconitase is characterized by a broad transition in the visible region near 410 nm. Extrusion of the Fe-S cluster has been performed in the presence of a large excess of extruding thiol (o-xylyl-(SH)<sub>2</sub> = o-xylyl- $\alpha, \alpha'$ -dithiol) in a protein denaturing medium (80% hexamethylphosphoramide). Core extrusion results in the formatioin of a  $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-o-oxyl})_4]^{2-}$  complex which

displays an absorption spectrum similar to native aconitase, but with a slightly increased extinction coefficient ( $\epsilon_{416} = 11,000 \text{ l/M}\cdot\text{cm}$ ) (Kurtz et al., 1979). The function of the iron-sulfur cluster in aconitase is not clearly understood. Ruzicka and Beinert (1978) have suggested that the Fe-S cluster may be the active center for aconitase action or is a sensor for the oxidation-reduction potential of the environment, which is involved in enzyme activation. It is interesting that aconitase is one of the few iron-sulfur protein not involved in a redox reaction.

Ribonucleotide reductase from calf thymus is activated nearly two-fold in the presence of exogenous iron.  $\text{Fe(III)Cl}_3$  and  $\text{Fe(II)-ascorbate}$  at a concentration of 60  $\mu\text{M}$  are equally effective in activity stimulation. Also, the presence of 6.2 mM DTE in the assay mixture causes quantitative reduction of the ferric to ferrous iron. Therefore, the activation conditions are analogous to these reported for aconitase. The iron content of homogeneous protein M2 is greater than that which could be accounted for, if the dimeric M2 subunit contained a single binuclear  $\text{Fe(III)}$  cluster as does E. coli protein B2. The electronic spectral properties of calf thymus protein M2 are significantly different from those of E. coli protein B2. However, the visible electronic spectrum of homogeneous M2 protein is comparable to the spectra of the Fe-S cluster in the oxidized form of aconitase and the  $[\text{Fe}_2\text{S}_2(\text{S}_2\text{-o-oxyl})_4]^{2-}$  complex (Kurtz et al., 1979). Finally, the power non-saturation characteristic of the protein M2 tyrosyl free-radical of hydroxyurea resistant mouse 3T6 cells suggests the presence of another iron center which is distinct from the putative antiferro-

magnetically coupled Fe(III) pair in protein M2 (Gräslund et al., 1982). Therefore, I propose that each M2 polypeptide of the mammalian ribonucleotide reductase may contain a  $(\text{Fe}_2\text{S}_2(\text{RS})_4)^{2-,3-}$  cluster, similar to that which occurs in aconitase. A corollary to this hypothesis is that the mammalian M2 subunit differs from the bacterial B2 protein in that it may contain two structurally dissimilar iron centers, instead of the single binuclear iron binding domain of the B2 protein.

Gräslund et al. (1982) have reported that incubation of hydroxyurea-inactivated ribonucleotide reductase with DTE (10 mM) under aerobic conditions leads to radical regeneration. Furthermore, the presence of  $\text{Fe}^{2+}$  (10  $\mu\text{M}$ ) in addition to DTE facilitates radical regeneration. In the B2 subunit of E. coli ribonucleotide reductase, the iron has to be removed and reintroduced as  $\text{Fe}^{2+}$  under aerobic conditions for regeneration to occur (Petersson et al., 1980). These investigators propose the regeneration of the mammalian enzyme radical species is possible since DTE can reduce  $\text{Fe}^{2+}$  in situ in protein M2 but not in protein B2. A subsequent reaction between  $\text{Fe}^{2+}$ , tyrosine, and oxygen would then generate both the  $\text{Fe}^{3+}$  pair and the oxidized tyrosine radical(s). If two tyrosine radicals are produced per iron pair, then the reaction stoichiometry is easy to account for.

The stoichiometric production of two tyrosyl radical species per protein M2 dimer is consistent with the hypothetical occurrence of one iron-sulfur cluster per M2 polypeptide, assuming that each polypeptide also harbors one radical species. A plausible reaction scheme for the

regeneration of the protein M2 tyrosyl free-radical(s) could be described as follows: 1) reduction of each  $[\text{Fe}_2\text{S}_2(\text{RS})_4]^{2-}$  cluster is carried out by DTE and facilitated (as in aconitase) by the presence of exogenous iron, 2) DTE also reduces the dimeric M2 subunit  $\text{Fe}^{3+}-\text{O}^{2-}-\text{Fe}^{3+}$  species to  $\text{Fe}^{2+}$ , 3) interaction of a specific tyrosyl residue with a reduced  $[\text{Fe}_2\text{S}_2(\text{RS})_4]^{3-}$  cluster in each M2 polypeptide resulting in residue oxidation would be accompanied by the simultaneous transfer of electrons (2) from the Fe-S clusters (one  $e^-$  per Fe-S cluster) to the  $2\text{Fe}^{2+}$  irons which 4) under aerobic conditions are oxidized to the  $\text{Fe}^{3+}-\text{O}^{2-}-\text{Fe}^{3+}$  moiety. In this example, the Fe-S cluster functions as a redox center in the facilitated transfer of electrons from tyrosines to the binuclear  $\mu$ -oxo bridged iron center. However, it is obvious that direct transfer may also occur. Thus, the function of the proposed Fe-S clusters may be as redox-indicators, which modulate enzyme activity less directly.

A protein M2-associated tyrosyl-radical signal similar to that reported for the bacterial enzyme was not detected in purified protein M2 or ribonucleotide reductase from calf thymus. This result is in agreement with a previous report (Thelander and Reichard, 1979). The presence of a paramagnetic center in the mammalian enzyme may explain why the protein M2-associated radical species has only been observed in intact cell preparations (Gräslund *et al.*, 1982). If the ribonucleotide reductase occurs as part of a multienzyme complex (Reddy and Pardee, 1980), then the enhancement of radical relaxation by interaction with a paramagnetic moiety may be lower in the "replicase" complex than that which occurs in a purified enzyme

preparation. Also, a greater relaxation rate for the tyrosyl radical species of the purified mammalian ribonucleotide reductase would cause the radical-specific EPR signal to broaden (and diminish) to the level background noise.

Thioredoxin is capable of replacing DTE in the regeneration of the protein M2-associated radical species. By analogy with the oxidation-reduction properties of protein B1, protein M1 may also participate in the reactivation process (Gräslund et al., 1982). However, thioredoxin has a regulatory function in photosynthesis, by mediating the transfer of reducing equivalents from chloroplast ferredoxin to acceptor regulating enzymes (Wolosiuk and Buchanan, 1977). It is therefore possible that thioredoxin reduces the ferredoxin-type iron center proposed for protein M2, and thus facilitates ribonucleotide reductase activation, in addition to any protein M1-specific interaction.

The hypothesis that the M2 subunit of the mammalian ribonucleotide reductase contains a ferredoxin-type iron-sulfur cluster provides a framework to guide further investigation as to the nature of protein M2-associated iron. Several different methods could be employed to demonstrate the occurrence, or lack, of an iron-sulfur cluster in protein M2. These methods would include 1) evaluation of  $S^{2-}$  release during sample acidification (Gillum et al., 1977), 2) displacement of the putative iron-sulfur center in the presence of an extruding thiol under protein denaturing conditions followed by spectrophotometric determination of the product complex (Orme-Johnson and Holm, 1978) and

3) evaluation of the EPR spectroscopic properties of homogeneous M2 at ultra-low temperature (13 K). Drawbacks to the EPR method include the necessity of working at ultra-low temperature (13 K) to slow the relaxation rate of the iron-sulfur center, possible interference in the spectrum due to the presence of other chromophores (tyrosyl-free radical), and a requirement for large amounts of sample (10-100 mg) (Chammack, 1975). Unfortunately, the method for protein M2 preparation described here yields only 0.5 mg of protein M2/1.5 Kg calf thymus. Thus, a logical starting point for further investigation would include modification of the protein M2 purification method to improve the yield of the protein. It is obvious that a number of questions pertaining to the nature of mammalian protein-M2 iron remain unanswered.

In conclusion, it is of interest to note the relationship of ribonucleotide reductase to other non-heme iron-containing proteins. In general terms, proteins containing non-heme iron can be categorized as either iron-sulfur proteins or those which contain iron ligated through non-sulfur amino acid side chains. In the former case, the iron-sulfur cluster usually mediates electron flow as is the case of rubredoxins and ferredoxins and/or in oxidation-reduction reactions involving enzymes containing other cofactors such as succinate dehydrogenase (contains FAD). The latter groups of non-heme iron containing proteins can be further delineated into 1) those proteins of iron storage (ferritin), iron transport (transferrin, lactoferrin, oxygen transport (hemerythrin) and 2) mixed-function oxidase type enzymes (oxygenases) and oxidoreductases. The oxygenases, such as

phenylalanine hydroxylase, in many cases have in addition cofactors other than non-heme iron. These include NAD, NADP, the flavinoids, iron-sulfur clusters, and heme (Bezkorovainy, 1980).

The absorption spectrum of ribonucleotide reductase (B2 subunit) from E. coli most closely resembles that of the oxygen transport protein, hemerythrin. Both proteins contain an antiferromagnetically coupled binuclear Fe(III) center. However, the role of the iron center in hemerythrin is to reversably bind oxygen, whereas the iron center in ribonucleotide reductase participates in the activation and stabilization of a second functional group, the tyrosyl free-radical. If the tyrosyl free radical species can be considered a "permanent" cofactor, then the enzyme should be classified as a complex non-heme iron-containing protein. Furthermore, if ribonucleotide reductase from mammalian sources also contains an Fe-S cluster, then this enzyme should be more properly categorized as a complex iron-sulfur protein (Orme-Johnson and Orme-Johnson, 1978).



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